MECHANISMS REGULATING NEUROMUSCULAR JUNCTION DEVELOPMENT AND FUNCTION AND CAUSES OF MUSCLE WASTING

Lionel A. Tintignac, Hans-Rudolf Brenner, and Markus A. Rüegg

Biozentrum, University of Basel, Basel, Switzerland; Department of Biomedicine, University of Basel, Basel, Switzerland; and INRA, UMR866 Dynamique Musculaire et Métabolisme, Montpellier, France

Tintignac LA, Brenner H-R, Rüegg MA. Mechanisms Regulating Neuromuscular Junction Development and Function and Causes of Muscle Wasting. Physiol Rev 95: 809–852, 2015. Published June 24, 2015; doi:10.1152/physrev.00033.2014.—The neuromuscular junction is the chemical synapse between motor neurons and skeletal muscle fibers. It is designed to reliably convert the action potential from the presynaptic motor neuron into the contraction of the postsynaptic muscle fiber. Diseases that affect the neuromuscular junction may cause failure of this conversion and result in loss of ambulation and respiration. The loss of motor input also causes muscle wasting as muscle mass is constantly adapted to contractile needs by the balancing of protein synthesis and protein degradation. Finally, neuromuscular activity and muscle mass have a major impact on metabolic properties of the organisms. This review discusses the mechanisms involved in the development and maintenance of the neuromuscular junction, the consequences of and the mechanisms involved in its dysfunction, and its role in maintaining muscle mass during aging. As life expectancy is increasing, loss of muscle mass during aging, called sarcopenia, has emerged as a field of high medical need. Interestingly, aging is also accompanied by structural changes at the neuromuscular junction, suggesting that the mechanisms involved in neuromuscular junction maintenance might be disturbed during aging. In addition, there is now evidence that behavioral paradigms and signaling pathways that are involved in longevity also affect neuromuscular junction stability and sarcopenia.

I. INTRODUCTION

The contractile activity of skeletal muscle is regulated by the central nervous system through the transmission of action potentials from motor neurons to muscle fibers. Transmission occurs at a highly specialized chemical synapse, the neuromuscular junction (NMJ) or motor endplate. Accordingly, impairment of NMJ function results in muscle weakness or paralysis. Muscle disuse impairs existing, and triggers new, signaling pathways in skeletal muscle, leading ultimately to severe muscle wasting.

Diseases that affect NMJ function are congenital myasthenic syndromes (CMS); autoimmune diseases, such as myasthenia gravis (MG) and Lambert-Eaton myasthenic syndrome (for review, see Refs. 124, 348); and various forms of intoxication, such as botulism. Other neuromuscular diseases are due to the death of motor neurons and thus loss of presynaptic input [e.g., spinal muscular atrophy (SMA) or amyotrophic lateral sclerosis (ALS)], to impaired myelination of the peripheral nerve [e.g., Charcot-Marie Tooth (CMT)], or to malfunctioning of the postsynaptic skeletal muscle fiber (e.g., muscular dystrophies). Severe forms of all these diseases are life-threatening, but fortunately, they are rare.

This review summarizes the current view of how the NMJ forms and is maintained, and how impairments in the neuromuscular system result in loss of muscle mass and function. The reader should keep in mind, however, that most of the mechanistic data available on this topic stem from experiments with rodents. Thus they should be adopted with caution to the human situation; for example, the clinical manifestation of a defect may be different for rodents and humans with their different loads on muscles associated with the different postures. Furthermore, it should be noted that we will not discuss all the pathways that have been implicated in neuromuscular disorders by genetic experiments, as the function of many of the respective gene products remains obscure. Thus current information may not go beyond the conclusion that those genes are “important” for a certain aspect of NMJ development or function without
II. PHYSIOLOGY OF NEUROMUSCULAR TRANSMISSION

The function of the vertebrate NMJ is to transmit nerve impulses in a 1:1 ratio from motor neurons to muscle fibers, thus subjecting the contraction of skeletal muscle to the control by the central nervous system. Both the presynaptic motor axon and the postsynaptic skeletal muscle fiber are highly specialized at the NMJ to ensure efficient transmission of action potentials (FIGURE 1). The NMJ has the characteristic structural features of other chemical synapses: the motor nerve terminal, packed with synaptic vesicles containing the transmitter acetylcholine (ACh), is separated from the postsynaptic muscle membrane by a narrow, 50- to 80-nm-wide gap, the synaptic cleft (FIGURE 1B). The postsynaptic membrane of the muscle fiber is deeply folded, with the crests of the folds carrying acetylcholine receptors (AChRs) at high density, and the troughs are equipped with high density of voltage-gated sodium channels (142; FIGURE 1B). The muscle fibers are tightly wrapped along their entire length by a basal lamina (also called basement membrane) containing extracellular matrix material originating from the muscle fibers. The basal lamina in the synaptic cleft differs in its molecular composition from that outside the synapse as it contains molecules secreted by both nerve and muscle (339). The nerve terminal is capped by specialized glial cells of the peripheral nerve, called terminal Schwann cells. Schwann cells also produce a basal lamina, which fuses with that of the muscle fiber at the edge of the NMJ. In addition, the poorly characterized kranocytes form a loose cover over the NMJ (103; FIGURE 1A).

For neuromuscular transmission of impulses, calcium influx associated with nerve action potentials in the motor nerve terminal elicits the fusion of synaptic vesicles with the terminal membrane at specialized sites, called active zones; vesicle fusion releases ACh into the synaptic cleft whose narrow width ensures its rapid (<1 ms) diffusion and binding to the AChRs in the subsynaptic muscle membrane to open their ion channels. AChRs are mainly permeable to sodium and potassium and, to a lesser extent, to calcium ions. The ACh content of one synaptic vesicle causes a net inward current of 3-4 nA into the muscle fiber, called quantal current or miniature endplate current (mEPC). A nerve action potential, by causing the simultaneous fusion of tens of vesicles, elicits a current of several hundred nA (at the muscle NMJ), which results in a local depolarization of the muscle fiber by −30–40 mV, called the endplate potential (EPP). The EPP is severalfold higher than required to reach the threshold for generating an action potential in the muscle fiber. This ratio is termed the “safety factor” of neuromuscular transmission (465).

The morphological and physiological properties of the NMJ that contribute to this safety factor are as follows:

1) The size of the nerve terminal, which determines the number of active zones.

2) The high density of voltage-gated calcium channels associated with the active zones that mediate calcium influx into the nerve terminal upon the arrival of the action potential and thereby trigger vesicle fusion.

3) The high concentration of ACh in a synaptic vesicle, which was estimated in the frog to be ~10,000 molecules/vesicle (239).

4) The number and density of the AChRs in the postsynaptic muscle membrane. While, in principle, the amplitude of the quantal current correlates with AChR density in the postsynaptic membrane, the relationship is not linear, if the AChR density is higher than needed for efficient capture of the ACh released (which is normally the case). As a consequence, a certain level of pharmacological blockade of AChRs reduces equilibrium currents in response to stable, nondesensitizing concentrations of exogenous ACh more strongly than mEPC amplitudes; for example, blockade or removal of ~80% of receptors is required to reduce the mEPC amplitude by 50% (340). Therefore, care must be taken in linearly extrapolating from data on receptor number and density to consequences for synaptic function.

5) The presence and depth of the postsynaptic folds with the high density of the AChRs at their crests and the high density of voltage-gated Na channels (Na1.4) in their troughs (142). This geometry and the high concentration of Na1.4 are important to ensure that the synaptic current generated through the AChR channels at the crests reliably triggers an action potential (401).

6) The high activity of acetylcholine esterase (AChE) associated with the synaptic portion of the muscle fibers basal lamina, which removes ACh rapidly from the synaptic cleft. This prevents repeated activation of individual AChR channels in response to a single action potential in the nerve.

Each of these parameters can be affected in CMS or MG (124, 348) and lower the safety factor, leading to weakness of muscle contraction.
III. DEVELOPMENT OF THE RODENT NEUROMUSCULAR SYSTEM

Neuromuscular transmission requires that pre- and postsynaptic components develop in tight register with one another, implying reciprocal interactions between the two. In this section we discuss the feedback between motor neurons and skeletal muscle fibers with an emphasis on how presynaptic factors can affect postsynaptic differentiation, and vice versa. The role of molecular components for NMJ maintenance is most readily resolved during NMJ development. Therefore, we start out with a description of NMJ formation and the roles of the molecules involved. We will focus on the rodent NMJ, since most of the concepts to be presented below are derived from experiments using either mice or rats. While numerous differences to human NMJs exist in their morphology and the time course of their development, molecular concepts derived from rodent NMJs have often been validated by the identification of the same genes being causative for acquired and congenital neuromuscular diseases in humans.
A. Development of Skeletal Muscle

Muscle fibers develop from progenitor cells in the paraxial mesoderm. In the trunk, paraxial mesoderm is segmented into somites that lie on either side of the neural tube. Myogenesis involves two waves of precursor proliferation: the first wave [embryonic day (E) 9.5 to E14.5 in mice] involves muscle progenitors that proliferate in the somites and migrate to their final location (e.g., limb) where most of them differentiate and fuse to form immature embryonic muscle fibers, called primary myotubes. A fraction of these progenitors, however, do not fuse and give rise to fetal myoblasts that continue to proliferate and either fuse with primary myotubes or, using them as a scaffold, begin to fuse among each other to form a new population of secondary myotubes between E15 and E17 (102).

The formation of normal numbers of secondary myotubes is dependent on innervation from the earliest stages of their development (13, 175). Moreover, the expression pattern of some muscle-specific genes is dependent on innervation (449, 450). For example, expression of AChR subunit genes and of distinct myosin isoforms that specify the contractile properties of the muscle (slow versus fast twitch) is strongly influenced by the pattern of impulse activity delivered by the nerve (388). Thus changes in presynaptic development affect innervation, which in turn can affect the development of the postsynaptic partner.

B. Development of the Diaphragm

Much of the work on the molecular mechanisms of synapse formation has studied NMJ development in the rodent diaphragm. Like other muscles, diaphragm in mice develops between E10.5 and about P10. The diaphragm forms from progenitors that are located within the pleuroperitoneal fold (PPF; Ref. 17). Primary fibers form between E11 to E12 (102), and as in other muscles, its formation precedes the pioneering phrenic axons from the cervical and brachial plexus, which arrive at the PPF at E12.5 in mice (54) and E13.5 in rat (3, 4). The axons, which later branch out from the main intramuscular nerve trunk, lag behind the first wave of muscle progenitors that migrate to either end of the diaphragm (FIGURE 2Aa). Myoblasts fuse to form primary myotubes only after they reach the full extent of the diaphragm and are subsequently innervated by multiple motor axons (115). Motor axons contact the developing hemidiaphragms at their midlines. Muscle progenitors destined for secondary myotube formation proliferate largely in the central region of the muscle, i.e., in the vicinity of the site of contact with the motor axons (FIGURE 2Ab). The enrichment of myogenic cells in the center of the diaphragm is reflected by a central, dense band of nuclei that express high levels of myogenin (17), a transcription factor essential for skeletal muscle development (177, 317). These secondary myotubes form beneath the basal lamina of the primary myotubes (FIGURE 2Ba). Around E14, motor nerves that innervate the primary myotubes progressively start also to innervate the secondary fibers (122; FIGURE 2Ba). At this stage, the primary and the secondary myotubes are electrically coupled (FIGURE 2Ba). As the secondary myotubes begin to elongate and separate from the primaries, they acquire their own basal lamina (FIGURE 2Bb; Refs. 222, 335).

After the transient, multiple innervation of muscle fibers (FIGURE 2Bb), each muscle fiber is innervated by a single motor neuron, and the highly specialized NMJ is established with the one-to-one match of pre- and postsynaptic specializations (FIGURE 2C; see also FIGURE 1B). The majority of muscle fibers in the adult muscle are derived from secondary myotubes. For example, in the adult rat diaphragm, they are the source of up to 80% of the muscle fibers (175). Importantly, their generation and maturation are strongly influenced by innervation and electrical activity. For example, in rat extensor digitorum longus (EDL), secondary myotubes require the presence of the nerve to develop their full complements at later stages (13) and to prevent atrophy and eventual degeneration (456). Moreover, in mutants in which ACh synthesis (ChAT deficiency; Ref. 304) or its impulse-evoked secretion (Munc-13 deficiency; Ref. 439) is lost, the muscle fibers of the diaphragm are severely atrophic, do not align, and mature, with many myotubes still containing centralized nuclei at E18.5. Thus the normal development of muscle requires functional innerva-
As a consequence, genetic manipulations that alter normal action potential activity in motor neurons or myotubes are likely to affect muscle development. Therefore, the defects in NMJ development in such mutants may be secondary to changes in normal muscle development rather than to a direct and specific effect on NMJ formation, making it difficult to interpret unequivocally the neuromuscular phenotype. Given this difficulty, it is surprising how little consideration has been given to this fundamental interdependence between nerve and muscle.

**FIGURE 2.** Development of skeletal muscle and innervation by motor neurons. A–C: overview of the different stages of development. The red-colored area around the center of the muscle indicates regions with high expression of myogenic factors and postsynaptic molecules, such as AChRs or MuSK. A: at early embryonic stages (E11 to E13.5), primary myotubes are formed, which largely precedes innervation by the motor nerve. A central zone of AChR clusters (red) without the contact to motor axons is formed. Aa: more detailed view of the initial phase of innervation. Primary myotubes contain some AChR clusters (red) and some myonuclei (gray) in their center express higher levels of Musk than those in the periphery (white). Ab: beginning of motor innervation of primary myotubes. The number of myonuclei with high levels of Musk (gray) increases, and myoblasts proliferate in the center inside of the basal laminae (brown) of the primary myotubes. The myonuclei of the proliferating myoblasts also express high levels of Musk and other myogenic factors. B: at late embryonic development (from E14.5 to early postnatal stages), muscle size increases by the formation of secondary myotubes. They form in the center of the developing muscle near the site of innervation. Ba: secondary myotubes are formed by the fusion of the proliferating myoblasts (shown in Ab) and become innervated. Innervation initiates transcription of synaptic genes in myonuclei (black) underlying the neuromuscular contact. Initially, the secondary myotubes are electrically coupled to the primary myotubes via gap junctions (indicated by the indentation of their sarcolemma). The developing secondary myotubes are still located within the basal lamina of the primary myotubes. Bb: secondary myotubes segregate from the primary myotubes and synthesize their own basal lamina (brown). Like the primary myotubes, they become multiply innervated, and electrical activity (flashes) leads to the restriction and condensation of AChRs to the site of innervation. At this stage, expression of synaptic genes is suppressed in nonsynaptic and selectively stimulated in the fundamental myonuclei. C: in mature muscle, each muscle fiber is innervated by one motor neuron.
Because of this problem, important concepts have also been derived from the study of so-called ectopic endplates formed in the adult animal. In this paradigm, proximal motor nerve stumps are surgically placed on the extrasynaptic region of adult, i.e., fully differentiated muscle, with motor axons subsequently growing in among the superficial muscle fibers without making synapses. When, 2–3 wk later, the muscle’s own nerve is cut, new, ectopic NMJs begin to form on the previously extrasynaptic membranes within 2.5–3 days, by a process that recapitulates all aspects of embryonic and fetal NMJ formation (for review, see Ref. 268). This experimental model offers the advantage 1) that the muscle fibers are fully differentiated and 2) that action potential activity in the nerve can be controlled separately from that in the muscle by selective pharmacological block or by electrical stimulation via implanted electrodes. Their respective effects on, e.g., postsynaptic differentiation or on synapse elimination can thus be investigated in a controlled way. An obvious disadvantage of this paradigm is that it is limited to genetic mutations that are not lethal.

C. Innervation of Muscle by Motor Neurons: Phenomenology

1. Embryonic to perinatal stages

Motor nerves reach the diaphragm at E12.5 in the mouse and form an intramuscular nerve trunk in the center of the muscle. At this time, AChRs form only a few, or no AChR clusters in the primary myotubes in the vicinity of the nerve fibers (136, 260), but they do not colocalize with them, similar to what has been observed in nerve-free myotube cultures (140; see also FIGURE 2A). One to two days later, neuromuscular contacts form in the center of the muscle, as indicated by the close apposition of nerve terminals with clusters of AChRs and AChE (260, 262, 471). However, this central band (∼200 µm wide) contains many AChR clusters that are not contacted by the nerve (FIGURE 2A); conspicuously, AChR clusters also form in mouse mutants lacking phrenic nerves (262, 471, 472), suggesting that receptor clusters are formed by a nerve-independent patterning mechanism. Hence, these aneural AChR clusters have been termed “prepatterned.” Between E13.5 and E15.5 (FIGURE 2, A AND B), the fraction of ACHR clusters contacted by the nerve increases from <10% to close to 50% (260). By E18.5, only innervated AChR clusters remain and have grown in size, while aneural clusters have disappeared (FIGURE 2B; Refs. 262, 471). In this early phase, a single postsynaptic AChR cluster may be contacted by up to 10 motor axons at birth (420). Importantly, the majority of AChRs are of the “fetal” subtype at these stages. Fetal AChR ion channels are composed of four different subunits, termed α, β, γ, and δ in the stoichiometry α2βγδ; they have an open burst duration of ∼4–5 ms (306).

2. Postnatal stages

The final steps of NMJ formation are accomplished in the first 2–3 wk after birth. During this time, multiple innerva-
tion of individual synaptic sites is reduced to a single innervating motor axon (60, 352, 400, 420). Moreover, expression of the fetal AChR channels along the fibers is largely lost. Beginning around the time of birth, fetal AChRs at the NMJ are gradually replaced by the adult subtype of AChRs over a period of ∼10 days and are further concentrated to high density (207, 288, 373). The adult AChRs contain the AChR ε-subunit instead of the γ-subunit, which gives rise to channels consisting of α2βεδ and results in shorter open burst durations (∼1 ms) but increased conductance for Na+, K+, and Ca2+ (306, 441). The physiological significance of this developmental switch remains largely unknown. A plausible, but unproven role of fetal AChRs for neuromuscular transmission in developing fibers could be that the average transfer of electric charge per open burst is larger and longer than for adult AChRs; as the time course with which fetal fibers polarize in response to the injection of step currents is longer than for postnatal fibers, spreading the current in time leads to greater depolarization (401). The AChR subunit switch does contribute, however, to the restriction of the endplate band to the central muscle region, but does not affect normal development of individual NMJs (231).

An analogous molecular switch occurs to the Na+1 channels in the postsynaptic membrane (272). Na+1 channels accumulate in the vicinity of immature rat NMJs at the time of birth (18). At this time, they are mostly of the Na+1.5 (tetrodotoxin-resistant) isoform (16). During the first two postnatal weeks, channels of the Na+1.4 (tetrodotoxin-sensitive) isoform become concentrated within the troughs of the developing postsynaptic folds. In contrast to the situation with AChRs, a detectable level of Na+1.4 channels persists in the extrajunctional membrane of adults to mediate the propagation of muscle fiber action potentials.

Other postnatal changes include the metabolic stabilization of synaptic AChRs (reviewed in Refs. 2, 374; see sect. VB2); the formation of subsynaptic infoldings of the membrane (280, 288); the recruitment of muscle nuclei, called fundamental myonuclei (up to 6 in adult mouse muscle; Ref. 238), to the cytoplasmic region below the synaptic membrane; and an increase in size and density of synaptic AChR clusters accompanied by changes of their shape from plaques to pretzels (400, 407). The breaking up of a plaque-shaped synaptic AChR cluster into a pretzel of the same area may be important for lowering the distance for which the synaptic current needs to flow across the high electrical resistance along the synaptic cleft, thus maintaining high driving force for the synaptic inward current. Finally, as the synaptic folds mature, AChRs become concentrated at their crests (139) and Na+1.4 in their troughs (142, 410).
Synaptic growth is functionally important for neuromuscular action potential transmission in older fibers. Because the amplitude of the synaptic potential evoked by the quantal current (mEPC) decreases as the fibers grow in size, muscle fiber growth requires compensation by an increase in synaptic current to maintain impulse transmission functional. Interestingly, consistent with the complementary roles of synaptic fold depth and density, and of synaptic growth for maintaining a high safety factor of neuromuscular transmission, these two parameters are inversely correlated in different species (465).

3. Synapse elimination

At birth, multiple (up to 10) motor axons connect to the same AChR plaque in every muscle fiber (60, 352, 420); in fact, in some muscles every motor neuron projecting to that particular muscle contacts each of its fibers (420). Within the first 2 wk of postnatal life, all but one of these inputs are lost, with a single axon per synapse remaining, a process called synapse elimination (400). For elimination, axons converging on the same synaptic site appear to compete with one another, and synaptic territory vacated by a loser is filled by a prevailing competitor (445). However, both the signals making some axons retract and those driving remaining axons to fill the vacated synaptic space are still debated. Based on the reversibility of “losing axons” in response to experimental ablation of prospective “winners,” some postulate that the primary withdrawal may occur at random (432), whereas others, using reinnervation paradigms in adult rodent muscle, have invoked synaptic activity and its timing in the competition process (see Ref. 134). Specifically, synchronous stimulation of motor axons converging on a synaptic site inhibits synapse elimination, both in the ectopic innervation paradigm (72) and at original synapses reinnervated upon nerve crush by original axons (132). Similarly, when a muscle receiving dual innervation via separate motor nerves (thus allowing their independent stimulation) is reinnervated upon complete denervation, elimination of supernumerary reinnervation from both nerves is inhibited if both nerves are stimulated synchronously (132). In contrast, elimination is promoted if the nerves are stimulated asynchronously (133). Consistent with a similar influence of impulse timing in newborn animals, motor neurons switch from synchronous to asynchronous firing around birth (64), probably as a consequence of reduced electrical coupling between them (341). Interestingly, it is at this stage when synapse elimination begins. It should be noted, however, that the molecular signals mediating axon retraction, whether spontaneous random or activity-dependent, remain elusive.

4. Role of Schwann cells

Schwann cells, which originate from neural crest cells, are the glial cells in peripheral nerves. They myelinate motor axons to increase the speed of impulse conduction, and they also cover the terminal arborizations of the motor axons at the adult NMJ (see Figure 1A). Schwann cell precursors formed at E12/13 from migrating neural crest cells give rise to immature Schwann cells at E13/15, which persist until the time of birth. Immature Schwann cells differentiate into myelinating and nonmyelinating Schwann cells, depending on whether they associate with large or small axons or with nerve terminals (terminal Schwann cells), respectively. The latter do not begin to myelinate the most distal presynaptic nerve branches until after synapse elimination has occurred (400).

The presence of Schwann cells is indispensable for normal innervation of muscle: mouse mutants lacking Schwann cells die perinatally, with defasciculating nerves showing aberrant muscle innervation patterns (263, 312, 353, 461, 462). Acute removal and pharmacological manipulation of terminal Schwann cells in frogs showed that terminal Schwann cells not only promote NMJ development, but are also required to maintain NMJ structure in the adult as well as its pre- but not postsynaptic function. Whether this effect is long-term (351) or acute (15, 357) remains unclear.

Terminal Schwann cells are also involved in synapse elimination. They not only destroy retracting, “losing” axon branches (45; see sect. III C3), but also attack winners and losers indiscriminately (402). They may thus be involved in the proposed random retraction of axons initiating synapse elimination (432), and they may drive synaptic turnover. Finally, terminal Schwann cells play essential roles during reinnervation of NMJs after nerve injury. By extending processes from denervated synapses they guide regenerating motor axons to vacant synaptic sites; in partially denervated muscle, Schwann cell processes extend from denervated to nearby intact synapses where they induce the surviving nerve terminals to sprout and grow back to denervated synaptic sites (404, 405). For more detailed accounts on the role of Schwann cells in NMJ development and function, the reader is referred to detailed reviews by others (110, 137, 413).

IV. MOLECULES IMPORTANT FOR NMJ DEVELOPMENT AND FUNCTION

Developing muscles express several molecules as part of their developmental program that are essential for synapse formation and function and later end up in the postsynaptic membranes. For clarity of the argument, we will group the molecules affecting NMJ development and function into two classes.

1) Proteins important for the reliable transmission of nerve impulses at the NMJ. These include the subunits of the AChR (α, β, γ, δ, ε), AChE, and the voltage-gated sodium channels (Na1.4 and Na1.5), which convert the EPPs into action potentials propagated along the muscle fiber.
Proteins that organize these components into a functional postsynaptic membrane. Here, we distinguish between “core proteins,” which are required for the development and maintenance of the NMJ, and “auxiliary proteins,” which contribute in different ways to full NMJ maturation and maintenance and thus can also affect the safety factor.

The “core proteins” include agrin, the signal derived from the innervating motor axon that is required for NMJ formation and maintenance, and proteins expressed in muscle fibers, such as muscle-specific kinase (MuSK), low-density lipoprotein receptor-related protein 4 (Lrp4), downstream of tyrosine kinases-7 (Dok-7), and 43 kDa receptor-associated protein of the synapse (rapsyn). The “auxiliary proteins” include neuregulins, AChRs, members of the epidermal growth factor receptor (ErbB) family, Wnts, and components of the dystrophin-glycoprotein complex (DGC).

All molecules discussed are schematically represented and functionally grouped in Figure 3.

A. Molecules Involved in the Neuromuscular Transmission of Action Potentials

1. AChR

During neuromuscular development, the muscle AChR subunit (Chrn) genes are first expressed when myoblasts fuse to form myotubes. Their expression is subsequently differentially modified by signals from the nerve both through molecular signals and by the electrical activity elicited in the muscle fiber. AChRs are localized at the crest of the synaptic folds (Figure 1B) through interactions with the DGC (Figure 3B).

![Figures A, B, and C](http://physrev.physiology.org/)

**FIGURE 3.** Schematic presentation of molecules involved in NMJ development. A: the agrin-Lrp4-MuSK-Dok7 complex is essential for the formation of the NMJ. Neural agrin with the proper amino acid inserts at the B/z site (indicated by the bulge in the most COOH-terminal domain) binds to the first YWTD repeat-containing β-propeller of Lrp4. Red double arrows indicate interactions mediating activation of MuSK. Other interactions are indicated by red arrows. Sites of phosphorylation in MuSK are shown (red dots). B: schematic of the structural components involved in the function of the NMJ. AChE is localized to the synaptic basal lamina and is essential to inactivate acetylcholine. The homotetrameric subunits, encoded by the Ache gene, coassemble with the triple helical collagen tail, termed ColQ, which tethers the entire enzyme to synaptic basal lamina. The dystrophin-glycoprotein complex (DGC) contains dystroglycan (DG), which is posttranslationally cleaved into α-dystroglycan (αDG) and the transmembrane component β-dystroglycan (βDG), the sarcoglycans (α through δ) and sarcospan (ss). DG associates with rapsyn to link AChRs to the DGC and connects to α-dystrobrevin (αDB), to α-syntrophin (syn) and to utrophin. Utrophin links the entire complex to the F-actin cytoskeleton. The voltage-gated sodium channels (Na,1.4) are localized to the troughs of the synaptic cleft (see also Figure 1). Interacting proteins (italics) and sites of interaction are indicated by red arrows. C: schematic presentation of the auxiliary components including neuregulins and their ErbB receptors, and the Wnts with their receptors Lrp5/6 and frizzled. For details on the nomenclature and the structure of the individual proteins, please see text and previous work and reviews (44, 66, 179, 293, 487).
The genes coding for the subunits composing the fetal AChRs (α, β, γ, δ) are first expressed in muscle precursors, and their expression is elevated when myoblasts fuse to myotubes. Transcripts encoding the AChR subunits accumulate in a central band of the developing muscle near the future region of innervation; conspicuously, centrally elevated transcripts are also seen in mutants that lack innervation (262, 471). Transcript enrichment is in register with the central muscle region where prepatterned, aneural AChR clusters form. As the formation of secondary myotubes is also initiated in the central region of the muscle (see also sect. III B), the spatial distribution of the Chrna mRNAs might be based on the high density of muscle precursors in this region (FIGURE 2, Ab AND BA), which express high levels of myogenin (17), a myogenic transcription factor also required for the constitutive expression of the fetal Chrna subunit genes (39, 40, 121, 298, 300, 346). Consistent with this, in neonatal leg muscle, Chrna1 (encoding the muscle AChR α-subunit) mRNA is expressed by many nuclei in a band around the newly formed synapses (57). Alternatively, centrally patterned Chrna mRNA may reflect myotubes with compartments of higher Chrna mRNA expression at their center than at their ends, perhaps regulated through Wnt signaling (see sect. IV B).

In late fetal and early postnatal development, the Chrna mRNA-rich band becomes increasingly condensed to the sites of innervation. Condensation of the Chrna subunit mRNAs to the synaptic sites may be the result of two simultaneous, but independent processes: the fusion of myoblasts with secondary myotubes, which comes to its completion around postnatal day 7 (FIGURE 2Bb), and the onset of nerve-induced action potential activity in the muscle fibers, which suppresses expression of α-, β-, δ-, and γ-Chrna subunits in extrasynaptic nuclei of the muscle fibers (160, 269).

As mentioned above, the basis of the developmental switch in AChR subtypes from “fetal” to “adult” at the synapse is a switch from the γ- to the e-subunit, resulting in a pentameric AChR with α2βδϵ stoichiometry. In contrast to the α-, β-, δ-, and γ-subunit mRNAs expressed in a band around the newly formed synapses, the e-subunit transcripts, from their earliest appearance just before birth, are always tightly associated with the nuclei directly underlying the synapse (57, 237). On a per nucleus basis, synaptic e-subunit mRNA levels increase strongly over a period of several days (57, 237). Over the same time period, the α-, β-, and δ-subunit mRNAs originally enriched in a perisynaptic band, become confined to the subsynaptic (fundamental) nuclei, where Chrna gene expression is resistant to nerve-induced muscle activity (237, 458). The fundamental nuclei in adult muscle thus express all the subunits required to form the adult AChR and thereby maintain functional impulse transmission in electrically active muscle. The association of activity-resistant Chrna subunit mRNA expression underneath the synapse strongly indicates that the innervating nerve provides the signal necessary to express those Chrna subunits in the fundamental nuclei (57, 458). Consistent with this idea, the Chrne (ε-subunit) is also expressed by the nuclei accumulated at ectopic endplates formed upon foreign nerve transplantation (56).

2. AChE

AChE hydrolyzes ACh released from the motor nerve terminal and thus terminates its action. It is highly enriched in the synaptic cleft through binding to the synaptic basal lamina and to MuSK. The elimination of ACh from the synaptic cleft is a very rapid process. This is indicated by the fact that the decay time constant of the mEPSCs is equal to or only slightly longer than the mean open burst duration of the synaptic AChR channels (99). This, in turn, suggests that following the release of an ACh quantum, AChR channels are only activated once, i.e., that ACh is hydrolyzed before multiple AChR openings by lingering ACh can occur. Accordingly, impairment of AChE activity will tend to increase the amplitude of the mEPSCs and to prolong their decay time. This principle is used therapeutically to ameliorate neuromuscular disorders caused by deficiency in synaptic AChRs, such as certain forms of CMS and MG. On the other hand, adequate AChE activity is essential for the maintenance of normal NMJs. Sustained activation of AChRs due to AChE deficiency causes excessive calcium influx through AChR channels and results in the activation of intracellular proteases such as calpains, which damages the postsynaptic membrane (251).

AChE at the NMJ is a very large protein (FIGURE 3B) consisting of three catalytic tetramers; each AChE module is derived from the same gene (Ache) which is expressed at elevated levels by the subsynaptic nuclei (209). Synaptic expression levels are further increased by action potential activity in the muscle; specifically, at ectopic endplates denervated at early stages of development exogenous muscle stimulation increases synaptic AChE (270).

The AChE tetramers are covalently linked in the Golgi apparatus to a noncatalytic AChE subunit, a three-stranded collagen-like tail, called ColQ (286, 361). Together with the synaptic expression pattern of Ache, ColQ mediates synaptic AChE enrichment both through binding to the proteoglycan perlecan, which is concentrated in the synaptic basal lamina (10), and to MuSK (82, 398). Accordingly, numerous mutations in the respective interaction domains of the COLQ gene have been identified in which AChE fails to localize to the NMJ, causing CMS with AChE deficiency (320, 333), and antibodies directed to the site in MuSK that binds to ColQ cause MG (221). These data clearly show the necessity of AChE and ColQ for NMJ function, yet they suggest that NMJs can form in their absence. Indeed, mice deficient for ColQ are born and breathe but fail to thrive and remain small. Death occurs at different time points in...
pressed along their entire length following denervation. In this context, the onset of muscle activity, and are reex-

Skeletal muscle fibers express two types of sodium channels, Na\textsubscript{\textalpha}1.5 and Na\textsubscript{\textalpha}1.4, with patterns revealing differential spa-
tiotemporal regulation (85). Na\textsubscript{\textalpha}1.5, which requires higher concentrations of tetrodotoxin (TTX) for blockade (there-

Agrin is the signal from motor neurons that organ-
zizes all aspects of the NMJ. Agrin is a heparan sulfate proteoglycan expressed by cells in different organs in different isoforms generated by tissue-specific, alternative mRNA splicing (FIGURE 3A; Ref. 44).

Subsequent cloning of the cDNA encoding agrin (369, 370, 431) and generation of mice with a targeted deletion of the Agrn gene (151) support the predictions of the agrin hy-

The Agrn gene is expressed in several isoforms as a result of alternative mRNA splicing (138, 369). The core protein has a predicted molecular mass of 225 kDa. Agrin is extensively N- and O-linked glycosylated at the NH\textsubscript{2}-terminal half, which lets the protein migrate on SDS-PAGE with an apparent molecular mass between 400 and 600 kDa (117, 430). Alternative mRNA splicing results in two different NH\textsubscript{2}-terminal ends; one isoform is expressed as a type II transmembrane protein (67, 69, 321), whereas the other isoform is secreted and encodes a binding site for laminins to allow its attachment to basal lamina (116, 218). While the transmembrane form of agrin is mainly expressed in neurons of the brain, motor neurons express mainly the secreted, laminin-binding form of agrin (44, 69, 321). Alternative mRNA splicing at the 3’ end of the transcripts generates protein isoforms that differ in the presence or absence of two small exons that encode 8 or 11 amino acids (138, 369). Thus splicing at this site (called B- or z-site) results in agrin isoforms with 0, 8, 11, or 19 (8 + 11) amino acid inserts. Importantly, isoforms lacking any amino acids at the B/z-site do not induce AChR clustering, do not acti-

B. Core Set of Proteins Required for the Formation of the NMJ

1. Molecules expressed by the motor neuron

A) AGRIN. Agrin is a giant

tent with the agrin hypothesis, motor neurons express agrin in nonsynaptic regions of a muscle is sufficient to create an ectopic and fully differentiated postsynaptic apparatus including folds that otherwise is only found at the NMJ (42, 94, 214, 294, 355, 410), as well as the synapse-specific expression of AChR subunit genes (214). These findings strongly support the notion that motor axons, by secreting agrin and contacting a muscle fiber responsive to agrin, initiate the formation of the NMJ (neurocentric model of NMJ formation).

Subsequent cloning of the cDNA encoding agrin (369, 370, 431) and generation of mice with a targeted deletion of the Agrn gene (151) support the predictions of the agrin hy-

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B) ACHR. AChR is a receptor for acetylcholine (ACh), which is released by motor neurons at the NMJ. The AChR channel is a pentamer composed of four subunits (alpha, beta, delta, epsilon), each with a transmembrane domain and a cytoplasmic region that contains the binding site for ACh. The alpha subunit is the most abundant and is responsible for the high conductance of the AChR channel.

C) SYNAPTIC PROTEINS. Synaptic proteins are a diverse group of proteins that provide the molecular machinery for the transmission of signals across the NMJ. These proteins include neurotransmitter receptors, ion channels, adhesion molecules, and cytoskeletal proteins. They are involved in the maintenance and function of the NMJ, including synaptic transmission, plasticity, and repair.

The synaptic portion of the NMJ contains specific synaptic proteins such as the presynaptic proteins, which are involved in the release of neurotransmitters from motor neurons. These proteins include the AChR, voltage-gated sodium channels, and calcium channels. The postsynaptic proteins, which are anchored to the sarcolemma of muscle fibers, include the AChR and the signaling molecules that regulate their activity. The synaptic basal lamina, which lies between the presynaptic and postsynaptic membranes, contains additional molecules that contribute to the formation and maintenance of the NMJ. These molecules include agrin, which is produced by motor neurons and acts as a signal to recruit AChRs to the synaptic terminal.

D) ACTIN. Actin is a filamentous protein that is a major component of the cytoskeleton. It is involved in the maintenance of cell shape, as well as in the regulation of muscle contraction and the proper localization of proteins at the NMJ. Actin is an abundant protein that is present in all cells, and it is involved in a wide range of cellular processes. In muscle cells, actin is organized into specialized structures such as the sarcomeres, which are the basic units of muscle contraction. In the NMJ, actin is involved in the formation of the synaptic fold, which is a key feature of the NMJ.

...
isoforms that include the amino acid inserts at the Bz-site, and skeletal muscle expresses the isoform lacking this insert (192, 369, 403, 431).

Deletion of Agrn prevents NMJ formation (151), and the mice are unable to breathe and thus die at birth. Recent evidence has implicated agrin also in the formation of muscle spindles (481). In early fetal diaphragm of agrin-deficient mice, a few dispersed AChR clusters are seen that are or are not contacted by nerve fibers (262, 471). This is similar to the AChR clusters transiently expressed in the central diaphragm region in nerve-free mouse mutants lacking the phrenic nerve (262, 471, 472). Agrn+/− mutants at E18.5 also lack a central band of elevated Chrna1 mRNA, which in wild-type mice colocalizes with the innervation region, consistent with a direct or indirect requirement of agrin for synapse-specific Chrna gene expression (151). Axons of the phrenic nerve in Agrn+/− mutants sprout excessively over the entire muscle, consistent with the lack of a stop signal provided by agrin itself (76, 77) and/or because of the lack of a postsynaptic specialization, which presents a retrograde signal to cause presynaptic differentiation. Interestingly, muscle-specific, transgenic expression of a miniaturized form of agrin that comprises only the NH2-terminal, laminin-binding region and the COOH-terminal region essential for AChR clustering is sufficient to rescue the Agrn+/− mice (261), arguing that it is not agrin itself that feeds back to the motor axon but a signal that is assembled during postsynaptic differentiation. Recent data have argued that Lrp4 can serve as such retrograde signal (see sect. IVB2).

2. Molecules expressed in muscle

A) MUSCLE-SPECIFIC KINASE. Muscle-specific kinase (MuSK) is a single transmembrane receptor tyrosine kinase that is involved in all aspects of NMJ development (FIGURE 3A). MuSK is the signaling component in the Lrp4-MuSK receptor complex necessary for triggering postsynaptic differentiation upon binding to neural agrin (227, 477). MuSK is required for the formation of aneural AChR clusters in embryonic myotubes (262) and for the formation of the postsynaptic apparatus, which clusters and anchors AChRs in the adult postsynaptic membrane (112). In addition, MuSK can recruit the fundamental myonuclei to the synapse and induce their transcriptional specialization (127, 215). Finally, it can, at least in part, induce a retrograde differentiation signal to the presynaptic motor axon (226).

Musk is expressed throughout muscle development, starting from early myotome (226). Upon innervation, Musk expression is downregulated in nonsynaptic muscle nuclei, but remains high in the fundamental nuclei, and is reinduced in nonsynaptic nuclei upon denervation (52, 437). Thus Musk mRNA expression follows the same pattern as Chrna subunit mRNAs. For example, Musk transcripts also accumulate in a prepattern (226), and it is thus likely regulated in a similar way as discussed above for Chrna subunits.

The most decisive evidence for the key role of MuSK in the formation of the NMJ came from knockout studies in mice (112). No AChR clusters are visible in any of the mutant muscles, and the mice die at birth because of respiratory failure. In addition, motor axons grow beyond the central muscle region along the entire myotube (112, 262, 471). Similarly, myotubes cultured from MuSK-deficient mice do not accumulate any spontaneous AChR clusters, nor do they respond to exogenous agrin (158).

MuSK activation is not only necessary, but also sufficient to induce the entire postsynaptic apparatus. This has been demonstrated by inducing dimerization and thereby self-activation of MuSK by overexpression (347, 376), by antibody-body-mediated crosslinking (195, 469) or by using a dimerization mutant in the transmembrane domain (215). In all those conditions, activated MuSK causes the formation of AChR clusters in cultured myotubes or, when expressed in nonsynaptic regions of the innervated skeletal muscle, it leads to the formation of nerve-free postsynaptic-like structures. Importantly, it also includes the accumulation of fundamental myonuclei that express genes encoding MuSK itself, ErbBs, and AChRs (215, 308, 376).

Self-activation of MuSK by overexpression in skeletal muscle is also able to induce some synaptic differentiation of motor axons in mice that are deficient for agrin (226). However, the mice are runted and survive only for a few weeks (226). The effect of MuSK on the motor axons may be indirect through the secretion of muscle factors acting retrogradely on the nerve terminal. For example, forced expression of constitutively active MuSK induces, at nerve-free ectopic postsynapses, the accumulation of the laminin-β2 chain (215), a laminin isoform that has been shown to regulate active zone formation during nerve terminal differentiation (327). Other components of the synaptic basal lamina that affect nerve terminal differentiation, such as fibroblast growth factors (FGFs) and type IV collagens (143), might be regulated similarly.

B) LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 4. Biochemical and genetic experiments provide strong evidence that low-density lipoprotein receptor-related protein 4 (Lrp4) associates with MuSK to form a receptor complex necessary for the binding of agrin (FIGURE 3A; Refs. 227, 466, 475, 477, 487). Specifically, Lrp4 has been shown to bind to agrin and to MuSK, thereby mediating tyrosine phosphorylation of MuSK by agrin (227, 477). The existence of such a coreceptor, at the time called muscle-associated specificity component (MASC), was postulated almost two decades ago (158) as agrin, when added to cultured myotubes, induced MuSK phosphorylation, but did not detectably bind to the MuSK ectodomain;
likewise, agrin did not induce MuSK phosphorylation in myoblasts or (upon forced expression) in nonmuscle cells (158).

The identification of Lrp4 as the agrin-MuSK coreceptor was largely guided by the serendipitous finding that mouse null mutants of Lrp4, generated chemically by using N-ethyl-N-nitrosourea (ENU), showed a NMJ phenotype with no AChR clusters and a failure of motor axons to stop at the presumptive synaptic band (451). In addition, cultured myotubes from Lrp4-deficient mice do not form AChR clusters upon addition of agrin. Thus Lrp4 deficiency largely phenocopies the neuromuscular defects observed in MuSK- and agrin-deficient mice. Biochemical studies later showed that 1) Lrp4, when coexpressed with MuSK in heterologous cells, confers agrin-induced MuSK phosphorylation; 2) the ectodomain of Lrp4 binds to the agrin isoforms expressed by motor neurons (neural agrin) but not to those expressed by nonneuronal cells (see details in sect. IVB1); and 3) Lrp4 binds to MuSK and this binding is enhanced by agrin (227, 477, 487).

The function of Lrp4 as the essential binding receptor for neural agrin has been corroborated by the cocrystallization of the first YWTD repeat-containing β-propeller of Lrp4 and the COOH-terminal laminin G-like domain (LG3) of neural agrin (containing an 8-amino acid-long insert at the B/z-site; see definitions in Ref. 44). This structure, resolved to 2.8 Å, revealed that the 8-amino acid insert (E-L-T-N-E-I-P-A) becomes highly structured in the cocrystal (487). In contrast, the structure of amino acid insert could not be resolved in a crystal of the LG3 domain of chick neural agrin with even higher overall resolution of up to 1.3 Å (408). These results therefore indicate an “induced-fit” binding between agrin and Lrp4. The interaction interface between the 8-amino acid insert and Lrp4 involves the middle portion of the insert (in particular asparagine and isoleucine in E-L-T-N-E-I-P-A) by forming hydrogen bonds with amino acids in Lrp4 (487). The structure data are in perfect agreement with previous site-directed mutagenesis of chick agrin LG3 showing that MuSK phosphorylation in cultured myotubes requires these amino acids (392). Most interestingly, a construct in which all, except the middle three amino acids of the insert, were mutated to alanine (A-A-A-N-E-I-A-A) has the same MuSK-activating potency as wild-type neural chick agrin (392), arguing that flanking amino acid solely serve as spacer to allow the “induced-fit” binding to Lrp4. The cocrystal between agrin and Lrp4 also contains agrin-agrin and Lrp4-Lrp4 interfaces, strongly suggesting that the functional agrin-Lrp4-MuSK complex is a dimer (FIGURE 3A).

At the NMJ, Lrp4 is expressed not only in skeletal muscle fibers but also in motor neurons, and this differential expression affects NMJ development in a distinct way. Selective deletion of Lrp4 in both skeletal muscle and motor neurons results in the complete abrogation of NMJ formation as observed after germline deletion of Lrp4 (466). In contrast, deletion of Lrp4 selectively in motor neurons does not result in any neuromuscular phenotype, and deletion in skeletal muscle alone still allows for the formation of NMJs so that the mice survive for weeks (466). These results are consistent with a model where the extracellular domain of Lrp4 is released from the cell surface of motor neurons and/or skeletal muscle fibers to form a functional agrin-Lrp4-MuSK complex (see FIGURE 3A). Indeed, Lrp4 knock-in mice that express only the extracellular domain of Lrp4 are indistinguishable from wild-type mice (118, 164), suggesting that transmembrane and intracellular domains of Lrp4 are not important.

The fact that mice deficient for Lrp4 in skeletal muscle show a presynaptic phenotype suggested that Lrp4 may also act as a retrograde signal for the motor axon. Indeed, presentation of Lrp4 to motor neurons in culture (either by forced expression of Lrp4 in nonmuscle cells or by immobilizing recombinant Lrp4 on polystyrene beads) is sufficient to induce differentiation of motor neuron growth cones into a presynapse-like structure (466, 475). Although those observations are intriguing, it is important to note that agrin and several additional components of the synaptic basal lamina membrane also induce the formation of presynapse-like structures in culture when presented on the surface of nonmuscle cells or when immobilized in the substrate (76, 77, 143, 199). Moreover, at the adult NMJ of the frog, synaptic basal lamina is alone sufficient to induce presynaptic differentiation (381). Thus it remains to be examined whether the Lrp4 ectodomain is found in synaptic basal lamina.

C) DOWNSTREAM OF TYROSINE KINASES-7. Downstream of tyrosine kinases-7 (Dok-7) belongs to a family of seven adaptor-like proteins (Dok-1 to Dok-7) that are characterized by NH2-terminal pleckstrin homology (PH) and phosphoryrosine-binding (PTB) domains followed by src homology 2 (SH2) target motifs (284). Several lines of evidence indicate that the Dok family members act as promiscuous cytosolic adapter molecules for tyrosine kinases. In some cases, binding of the Dok protein inhibits tyrosine kinase activation, whereas in others (as is the case for Dok-7), binding strongly enhances tyrosine kinase activity (284). Similar to the discovery of Lrp4 being the coreceptor of MuSK, the identification of Dok-7 as an adapter necessary for the full activation of MuSK was based on the observation that mice deficient for Dok-7 die perinatally because of respiratory failure due to the lack of NMJs (334). Similar to agrin-, MuSK-, or Lrp4-deficient mice, motor axons overshoot and grow over the entire muscle fiber. In contrast to Lrp4, expression of Dok-7 is confined to skeletal and heart muscle (334), and thus the lack of Dok-7 may explain the failure of agrin to activate MuSK in nonmuscle cells (158).
The binding of Dok-7 to the cytoplasmic region of MuSK resides in the NPYX sequence motif at the juxtamembrane region whose phosphorylation at the tyrosine residue is necessary and sufficient for MuSK activity (Figure 3A; Refs. 184, 185). Cultured myotubes from Dok-7 knockout mice do not form AChR clusters upon addition of agrin, nor is MuSK activated, indicating that Dok-7 is required for MuSK function. Activation of Lrp4-MuSK by agrin also causes phosphorylation of Dok-7, which in turn, creates binding sites for v-crk sarcoma virus CT10 oncogene homolog (Crk) and Crk-like (Crk-L) (172). Skeletal muscle-specific deletion of Crk and Crk-L results in smaller NMJs and aberrant innervation and mice die around birth (172). Crk and Crk-L are widely expressed and interact with many intracellular kinases, including c-abl oncogene 1 (Abl). Moreover, germline deletion of Crk alone is embryonic lethal mainly because of vascular issues resulting in bleeding and malformations of the heart (337). Thus it remains an open question of how specific the interaction of Crk and Crk-L is for NMJ signaling.

Another rather unusual aspect of Dok-7 signaling is the finding that forced overexpression of Dok-7 is sufficient to form AChR clusters in cultured myotubes and to allow mice to survive for several weeks in the absence of agrin (334, 422). Similarly, expression of Dok-7 via AAV vectors in skeletal muscles enlarges the NMJ and thereby ameliorates the disease in a mouse model for Emery-Dreifuss muscular dystrophy (11). These findings show that Dok-7 can also mediate inside-out signaling, a phenomenon that is well documented for integrins, a family of heterodimeric (with one α and one β subunit) receptors that bind to a wide variety of extracellular matrix proteins (278). Interestingly, the Dok-7 homolog Dok-1 binds to the NPXY motif of integrin β-subunits and inhibits the inside-out activation of the integrins (6). Thus MuSK and integrin activation could be a result of convergent evolution.

D) 43 KDA RECEPTOR-ASSOCIATED PROTEIN OF THE SYNAPSE (RAPSYN). Rapsyn is a cytoplasmic scaffolding protein expressed constitutively in myotubes; it is present at the NMJ from the earliest stages of development, and in adult muscle, expression is largely restricted to the synaptic region. Rapsyn binds tightly to AChRs to form a high-density network of the two proteins (434). Rapsyn also binds to dystroglycan (26) and is thus thought to link AChRs to the postsynaptic actin cytoskeleton (Figure 3B). The molecular signaling pathways that lead from MuSK activation to the clustering of the AChR-rapsyn network are not well understood.

Rapsyn is required for AChR cluster formation in cultured myotubes and for NMJ formation in vivo. Accordingly, mice deficient for Rapsn fail to form postsynaptic specializations (152). Interestingly, Rapsn knockout mice have a less severe phenotype than MuSK-, Lrp4-, or Dok-7-deficient mice as they are able to breathe weakly and therefore survive for a few hours after birth. Examination of the neuromuscular contact sites revealed lack of any discrete AChR clusters and cytoskeletal specializations. However, components of the synaptic basal lamina, such as AChE and laminin-β2, still accumulate at the synapse. Interestingly, MuSK is still aggregated underneath the nerve terminal (8), and ChRNA1 mRNA is highly expressed in the fundamental myonuclei (152). These experiments show that rapsyn is essential for the tethering of AChRs to the postsynaptic apparatus but not for synaptic gene transcription; furthermore, they imply that at early postnatal stages even non-clustered AChRs at the neural contact site are sufficient to support some low-level muscle contraction.

E) AChR. In addition to its role in neuromuscular transmission, AChRs play an active role in the assembly of the postsynaptic apparatus. Specifically, unlike what has been described for nonmuscle cells where rapsyn becomes clustered when overexpressed (e.g., Ref. 343), the formation of such clusters in muscle cells requires AChRs. For example, antibody-mediated depletion of AChRs from the cell surface of cultured myotubes or low-power laser-induced removal of AChRs results in the concomitant loss of rapsyn clusters (62, 276). Moreover, rapsyn has been found to assemble with AChRs already in the secretory pathway (277, 310). The active role of AChRs in the formation of the postsynaptic scaffold is also supported by the finding that deletion of the gene encoding the AChR γ-subunit (Chrng), which largely prevents the assembly of functional AChRs during embryogenesis, results in continued growth of motor axons beyond the synaptic band and in aberrant nerve branching (264). Interestingly, MuSK is still clustered in the middle of the diaphragm in those mice, whereas rapsyn remains unclustered. In summary, AChRs seem to contribute to postsynaptic differentiation, and functional AChRs are important for the delivery of rapsyn to the postsynaptic apparatus.

C. Auxiliary Proteins That Contribute to NMJ Formation

1. The dystrophin-glycoprotein complex

The structural specializations of the postsynaptic membrane, including its high AChR density, are maintained by the subsynaptic apparatus, an elaborate, specialized network of the cortical actin cytoskeleton and comprising, in addition to MuSK and rapsyn, the dystrophin-glycoprotein complex (DGC; Figure 3B). The DGC is a transmembrane complex of proteins linking the actin cytoskeleton of the muscle fiber to the basal lamina. It includes dystrophin or its homolog at the synapse, utrophin, linked to cytoskeletal actin, three groups of transmembrane proteins dystroglycan (DG), sarcoglycans and sarcospan, and two groups of cytoplasmic proteins, the dystrobrevins and syntrophins (399). In nonsynaptic muscle regions, the DGC is required to prevent muscle fiber damage caused by contraction, as
shown in muscular dystrophies, which often result from mutations in components of the DGC (111). Dystroglycan is posttranslationally cleaved into the transmembrane β-dystroglycan and the extracellular, sarcolemma-associated α-dystroglycan (202). α-Dystroglycan is heavily glycosylated, and this glycosylation is essential for the binding to its ligands agrin, laminins, perlecan, neurexin, and pikachu-rin (51, 126, 385, 412, 416). Accordingly, mutations of the enzymes that are involved in the glycosylation of these unusual sugar side chains cause underglycosylation of DG and can result in very severe, early-onset congenital muscular dystrophies (309).

At the NMJ, the function of α-dystroglycan as a binding receptor for agrin is not well established. Initial reports that suggested α-dystroglycan being the agrin receptor important for AChR aggregation (51, 78, 153) proved to be incorrect as α-dystroglycan binds to the agrin isoforms that lack amino acid inserts at the β/ε site much more strongly than those that carry an insert at this site (155, 414). Moreover, the agrin deletion fragment that is sufficient to induce AChR aggregation (156) and rescues the NMJ phenotype of agrin knockout mice (261) does not bind to α-dystroglycan at all (155). However, recent genetic manipulations of the extent of glycosylation of α-dystroglycan have shown that underglycosylation causes fragmentation of the NMJ (159, 174), indicating that dystroglycan has a stabilizing role for the NMJ. In addition, deletion of intracellular components of the DGC impairs postnatal (but not neonatal) synaptic development, including AChR stability (AChR half-life), AChR density, and fold formation (2, 167). For example, deletion of α-dystrobrevin or syntrophin, in addition to impairing NMJ structure, reduces AChR density and lowers AChR half-life by reducing AChR recycling (see sect. VB2; Refs. 165, 282). Similar, although weaker, effects are generated by mutations in α-dystrobrevin, which prevent COOH-terminal tyrosine phosphorylation (165), or in α-syntrophin (282). A severe fragmentation and loss of the NMJ is only observed in triple mutant mice that lack utrophin, dystrophin, and α-dystrobrevin (167), whereas single deletions have only mild effects (113, 166, 167, 273). Thus it is difficult to attribute specific functions to α-dystrobrevin, dystroglycan, or syntrophin for the stability of the NMJ.

2. Neuregulins

The neuregulins (Nrgs) (FIGURE 3C) have a domain in common with epidermal growth factor (EGF). There are six closely related neuregulin genes Nrg1 to Nrg6 (293). Isoforms of Nrg-1 and Nrg-2 are expressed by motor neurons, muscle cells, and/or terminal Schwann cells and are recruited to the postsynaptic muscle membrane (295, 313, 355, 356), but their functions in NMJ development in vivo are poorly understood.

Among the Nrg-1 isoforms, Nrg-1β, originally identified as acetylcholine receptor inducing activity (ARIA), was isolated from chicken brain based on its ability to increase expression of Chrna1 mRNA in cultured chick myotubes (130, 211). It is expressed in motor neurons and was proposed to be the motor neuron released factor to control expression of Chrm subunits genes in fundamental myonuclei (see sect. VB1). Deletion of Nrg1 is lethal due to heart malformation (302). However, while Nrg1 mutants can be rescued by transgenic expression of Nrg-1 in the heart, neuromuscular development remains impaired, apparently through impairment of Schwann cell development and survival (250, 428; see also sect. III C4) rather than through impaired signaling in skeletal muscle (127). However, Nrg1 deletion abolishes normal muscle spindle development (191).

3. ErbB receptors

The founding member of the ErbB family of receptors is the epidermal growth factor receptor (EGFR; also called ErbB1). ErbB proteins are receptor tyrosine kinases comprising ErbB2, ErbB3, and ErbB4 isoforms, which are encoded by distinct genes. They share high similarity with the EGF receptor and are stimulated by Nrgs through dimerization. However, ErbB2 lacks the binding site for Nrg-1, ErbB3 lacks a kinase domain; thus signaling occurs through homo- (ErbB4) or heterodimerization in the combinations ErbB2/3, ErbB2/4, or ErbB4/EGFR (452). Dimerization leads to auto- and trans-phosphorylation of tyrosine residues in their intracellular domains, which serve as docking sites for adaptor proteins and enzymes initiating downstream signaling cascades, including the Raf-MEK and JNK pathways.

ErbB2, -3, and -4 are also expressed in the postsynaptic muscle membrane (429). Like Musk, Chrn subunits, and Rapsn, Erbb2 and -3 are expressed at increased levels from fundamental nuclei in response to MusK activation (308). Selective depletion of ErbB2 in skeletal muscle destabilizes synaptic AChRs and lowers their density in the postsynaptic muscle membrane, apparently through impairment of α-dystrobrevin function (389; see also below). Also, it impairs normal muscle spindle development (252). In contrast, germline deletion of Erbb2 in mice, in which the malformation of the heart was prevented by transgenic expression of Erbb2, causes aberrant development of motor axons and NMJs, apparently through impairment of normal Schwann cell development (263, 461; see also sect. III C4).

4. Wnts

Wnts are secreted glycoproteins expressed as 15 isoforms in zebrafish and as 19 isoforms in mouse and humans. They are involved in multiple aspects of development (179, 438), including axon pathfinding and synaptogenesis. Their function during the formation of the NMJ has been best de-
scribed in invertebrates (Drosophila melanogaster and Caenorhabditis elegans), and involvement of Wnt signaling at the vertebrate NMJ has also been revealed (reviewed in Refs. 24, 232). Wnts in mammals signal through 11 different Frizzled (Fzd) receptors to regulate, via at least five different transduction pathways involving Dishevelled (Dvl), various cellular processes (Figure 3A).

The strongest evidence for the physiological function of Wnt signaling in the neuromuscular system in vivo stems from work in zebrafish, which indicates a role in the patterning of muscle innervation. As described above (sect. III C1; Figure 2), AChRs cluster in the presumptive innervation zone in the center of the developing muscle independently and prior to the appearance of motor axons. In developing zebrafish, deletion of the unplugged gene, the ortholog of mammalian Musk, abolishes AChR prepatterning, impairs axon guidance, possibly due to modifications in the extracellular matrix, and NMJ formation (391, 480). Deletion of SV1, which is one of the three unplugged splice variants that lacks the Ig domains required for MuSK to be phosphorylated by agrin (484), abolished AChR prepatterning and results in aberrant axon branching of the motor neurons (480). Interestingly, however, the formation of NMJs is still possible when expression of full-length unplugged is again restored after the initial loss of the prepatterning (212). Thus prepatterned AChRs are dispensable for NMJ formation, consistent with the observation in mouse diaphragm that NMJs can also form outside the zone of prepatterned AChR clusters (260). The guidance of motor axons to the central muscle zone and the prepatterning of AChRs acts in zebrafish via the binding of unplugged/Musk to Wnt11r (212). Taken together, these findings indicate that unplugged/Musk, activated by Wnt11r, confines motor axon outgrowth and promotes AChR prepatterning in the central muscle region. This region, in mouse diaphragm, is also most sensitive to agrin (260).

The mechanisms by which Wnt signaling affects prepatterning at rodent NMJs is far from understood. In addition, given the involvement of Wnts in myogenic proliferation and differentiation (443), Wnts may also affect the innervation pattern indirectly by altering muscle differentiation (see sect. III B). For example, Wnts might affect the elongation of secondary myotubes in developing muscle, which according to the model presented in Figure 2 (see sects. IIC and IVA), would affect the width of the prepatterned muscle region. Alternatively, Wnts might affect expression of molecules involved in synapse formation, such as MuSK (224) or rapsyn (447), thus modulating agrin responsiveness. Experiments in mouse diaphragm are consistent with several of these possibilities. For example, genetic deletion of Wnt4 widens the central band of prepatterned AChR clusters in developing muscle, reduces their numbers, and causes nerve overgrowth (411); a function similar to that described for Wnt11r in zebrafish (212).

Experiments aimed at better understanding the molecular mechanisms of how Wnts affect prepatterning have focused on 1) testing binding of different Wnts to MuSK and 2) examining their effect on AChR clustering in cultured myotubes. These experiments, while failing to yield a conclusive picture, have indeed provided evidence that several Wnts bind to MuSK and affect AChR clustering. For example, most Wnts tested bind to the extracellular, frizzled-related, cysteine-rich domain (CRD) of MuSK (411, 476; see also Figure 3A), a site that is distinct from the Ig domains, which have been implicated in mediating agrin-induced activation of MuSK (484). Moreover, autoantibodies directed towards MuSK that cause MG can also be directed to the CRD domain of MuSK (415). Finally, AChR clustering assays on cultured myotubes indicate that Wnts promote MuSK phosphorylation in the absence of agrin (411, 476) or enhance AChR clustering induced by agrin (183). The Wnts implicated in those activities are Wnt-3, Wnt-4, Wnt-9a, and Wnt-11 (24). However, other Wnts have recently been shown to inhibit agrin-induced AChR clustering in culture (24).

A role of Wnts in NMJ formation and maintenance is further corroborated by the findings that components of the Wnt pathway, as for example Dvl1, bind to the intracellular domain of MuSK (271) and that deletion of the Wnt components β-catenin (253) or adenomatous polyposis coli (APC; Ref. 446) perturb aspects of NMJ formation in rodents. In addition, mice deficient for the gene encoding caspase-3, which cleaves Dvl1, show a transient increase in the number of aneural AChR clusters in the developing mouse diaphragm (448). However, the mice do not show any major NMJ phenotype postnatally, arguing that the interaction of MuSK with Dvl1 is of minor importance for NMJ function. Finally and most importantly, knock-in mice in which full-length MuSK is replaced by a mutant that lacks the CRD domain (MuSKΔCRD) show an aberrant prepatterning during development and exert a CMS-like phenotype including weakness and fatigability (301). In summary, Wnts appear to have a role at the NMJ, and some of those effects seem to be mediated by their interaction with MuSK. However, the detailed mechanisms involved are still not understood; one difficulty in resolving them could be the redundancy and promiscuity of the different Wnt isoforms.

V. REGULATION OF SYNAPTIC AChR CLUSTER FORMATION

A. Regulation of AChR Cluster Formation in Fetal Muscle

As mentioned above, starting at E12, constitutively expressed AChRs begin to cluster in a central band of fetal diaphragm independently of innervation (262, 471, 472). Aneural AChR cluster formation is dependent on the expression of MuSK and of some signaling molecules down-
stream of MuSK, such as Dok-7. Given that MuSK and Dok-7 can induce AChR clustering in the absence of agrin by forced overexpression, the pre patterning of AChRs may reflect autoactivation of MuSK signaling in the oldest, central region of the developing muscle.

It has been proposed that the muscle-autonomous, aneural AChR clusters serve as potential platforms for the nerve to form NMJs (241). In this model, secretion of agrin by the nerve selectively stabilizes the preexisting AChR clusters it contacts while AChR clusters remaining without neural contact would be dispersed through the secretion of ACh from the nerve terminals (241). Importantly, in this model, it would be the muscle, through the generation of prepatterned AChR clusters, that determines the exact location for a new NMJ to be formed; hence, this model is known as the myocentric model of NMJ formation. The myocentric model thus abandons the agrin hypothesis where the nerve, by secretion of agrin, determines the site of a synapse (neurocentric model; Ref. 290).

While the two models each represent extreme cases, the true mechanism of how NMJs form may in fact include both views. For example, in zebrafish larval muscle, where NMJ formation can be observed in real time, a majority of motor axons grow and extend their filopodia preferentially towards preexisting AChR clusters, where they begin to accumulate nerve-terminal-specific material, as predicted by the myocentric model (336). In contrast, other axons induce new AChR clusters and may also incorporate preexisting AChR clusters (141). Interestingly, the formation of presynaptic specialization in the central region of the muscle was still observed when AChRs were blocked by α-bungarotoxin (α-BTX) or when the formation of AChR clusters was abolished by genetic ablation of the δ-AChR subunit (336). Thus muscle-derived cues that are colocalized with, but do not represent, the prepatterned AChR clusters themselves determine motor axon outgrowth, branching, and initial neuromuscular synaptogenesis. Support of the neurocentric model is provided by observations in mouse diaphragm and in zebrafish (212, 260) where NMJs also form outside the normal endplate band. Indeed, at E12.5, representing the time when nerves make first contacts with the diaphragm, the phrenic nerve of ChAT−/− mice already shows exuberant branching compared with control mice (54). As development proceeds, both aneural and nerve-associated AChR clusters are observed, but the “synaptic” band is much wider and the number of AChR clusters is higher than in control mice (54, 304). Moreover, ChAT deficiency also affects motor neuron survival (many more motor neurons are detected in the spinal cord) and myogenesis (muscle remains much thinner), consistent with the observation that in culture, ACh expressed by muscle cells is required for fusion of myoblasts to form myotubes (125, 236).

An alternative interpretation of the genetic data from ChAT/agrin double mutants would be that the lack of ACh, by blocking neuromuscular transmission, impairs muscle development and upregulates synaptic genes, such as AChR and MuSK, thus increasing the agrin responsiveness of muscle regions outside the normal endplate band. Indeed, at E12.5, representing the time when nerves make first contacts with the diaphragm, the phrenic nerve of ChAT−/− mice already shows exuberant branching compared with control mice (54). As development proceeds, both aneural and nerve-associated AChR clusters are observed, but the “synaptic” band is much wider and the number of AChR clusters is higher than in control mice (54, 304). Moreover, ChAT deficiency also affects motor neuron survival (many more motor neurons are detected in the spinal cord) and myogenesis (muscle remains much thinner), consistent with the observation that in culture, ACh expressed by muscle cells is required for fusion of myoblasts to form myotubes (125, 236).

B. Stabilization of NMJs in Postnatal and Adult Muscle

The fact that several synaptic components remain expressed in the fundamental myonuclei after the assembly of the NMJ while they are gradually downregulated in extrasynaptic regions by electrical muscle activity implies that their synapse-specific expression is maintained in an activity-resistant manner by signals from the nerve. Indeed, in situ hybridization shows that mRNA levels encoding these and several other components are higher at the NMJ than outside. The mechanisms important for the maintenance of expression in the fundamental nuclei have been most extensively studied for the Chrm subunit genes.
1. Regulation of Chrn gene expression at the synapse

Several molecules from the nerve have been implicated in mediating the neural regulation of synapse-specific Chrn and Musk gene expression in fundamental nuclei. Among them, Nrg-1β (ARIA; Refs. 130, 211) and agrin have commanded most interest.

Nrg-1β is present at the NMJ as well as at agrin-induced ectopic postsynaptic membranes (295, 354), implicating its recruitment also from muscle. Its receptors, the ErbBs, are enriched in the postsynaptic muscle membrane (486). However, in adult mice in which Nrg signaling to muscle was abolished by muscle-selective deletion of ErbB2 and ErbB4 receptors, the NMJs are only marginally affected, and synaptic transcription of the Chrne, Chrnd, and Musk genes at NMJs is grossly normal (127, 242). Thus Nrgs are dispensable for synapse-specific Chrn gene transcription in vivo.

Instead, synaptic genes appear to be regulated by agrin. Specifically, ectopic expression of agrin or of a constitutively active form of MuSK in muscle in vivo induces ectopic, nerve-free postsynapses, including the transcription of the η-subunit of the Chrn and of Musk genes; this occurs even in the absence of Nrg/ErbB signaling (127, 242), indicating that agrin–MuSK signaling not only triggers aggregation of AChRs to the site of nerve-muscle contact but also directs the transcription of genes encoding AChRs and, in a positive-feedback loop, the gene encoding MuSK (308).

How is agrin-induced transcription of these genes mediated? Several of the synaptic genes, including Chrne, Chrnd, Ache, utr (utrophin), and Musk, share a regulatory, six-base-pair-long sequence, termed N-box, that targets their expression to subsynaptic nuclei (see review in Ref. 386; FIGURE 4A). Specifically, when introduced in vivo into muscle fibers in the context of the respective promoter-reporter fragments, N-boxes confer reporter expression to the synaptic region, but with lower spatial specificity compared with the expression of the endogenous gene product; single point mutations of the N-box abolish synapse-specific transcription, but not upregulation in extrasynaptic nuclei upon denervation (144). Combined, these data strongly suggest that the N-box is required for synapse-specific transcription, perhaps in cooperation with other promoter sites. Independent confirmation of its physiological relevance comes from patients who suffer from severe AChR deficiency (a form of congenital myasthenia) that is associated with point mutations in the N-box of the Chrne promoter (322, 332).

The pathways linking agrin-Lrp4-MuSK and N-box-dependent activation of synaptic genes remain uncertain. Factors proposed to mediate the transcriptional response of Chrn and Musk genes from subsynaptic nuclei include GA-binding protein GABPa/GABPβ dimers (58, 144, 387) and Erm (190), both members of the Ets family of transcription factors (FIGURE 4A). Of these, GABPa/GABPβ indeed binds to the N-box upon Nrg-1-induced phosphorylation of GABPa in cultured muscle cells (144), and forced expression of a dominant-negative mutant of GABPβ in muscle fibers inhibits agrin-induced postsynaptic differentiation in vivo (58). However, disruption of GABPa in vivo does not or only marginally affects NMJ function, consistent with GABPβ binding to the N-box, but arguing against a physiological role in synapse-specific transcription (210, 328). In contrast, deletion of the Ets transcription factor Erm strongly impairs normal NMJ formation and function in vivo, and is associated with downregulation of many synaptic genes; however, binding of Erm to the N-box sequences has not been tested directly, and its disruption is not lethal, indicating that Erm boosts, but does not regulate synaptic genes in an all-or-none manner (190). Finally, experiments in cultured muscle cells suggest that agrin-Lrp4-MuSK signaling can activate N-box-dependent gene expression via ERK and JNK pathways (242).

In summary, the signaling pathways discussed above maintain expression of Chrn subunit genes from the fundamental nuclei at the synapse against their repression by electrical muscle activity, as occurs in extrasynaptic nuclei. The latter is mediated by the increase in cytosolic calcium through the activation of L-type calcium channels associated with muscle stimulation. For details of activity signaling in Chrn subunit expression, the reader is referred to the review by Sanes and Lichtman (380).

2. Regulation of synaptic AChR density through AChR insertion and removal

In fetal muscle AChRs become clustered by agrin-Lrp4-MuSK signaling (154, 296). Experiments examining AChR clustering induced by the addition of neural agrin in cultured muscle cells indicate that synaptic components (including AChRs, rapsyn, and MuSK) are delivered to the cell surface via exocytic vesicles (277). While AChR, MuSK, and rapsyn appear to be constitutively associated with lipid rafts, stimulation with agrin and subsequent activation of MuSK results in their coalescence first into AChR microclusters and then into larger clusters (75, 338, 485). As a consequence, disruption of lipid rafts inhibits agrin-induced AChR clustering. Microcluster coalescence is thought to be driven by actin-based raft movements promoted by local actin polymerization induced in turn through agrin-Lrp4-MuSK-induced activation of small GTPases Rac, Cdc42, and Rho (455) as well as the actin nucleation factors N-WASP and the Arp2/3 complex (81). Indeed, Rac is involved in the formation of AChR microclusters, whereas Rho seems important for their coalescence to large aggregates (454). Finally, recent studies described Coronin 6 as being associated in AChR clusters and mediating their association with the actin cytoskeleton (87). Although in vivo knockdown of Coronin 6 by electroporation of shRNA...
results in the fragmentation of NMJs, it remains to be shown how Coronin 6 mediates the interaction with the actin cytoskeleton and whether there is any connection to the DGC, which has also been implicated in the linkage of AChRs to the actin cytoskeleton (see sect. IV C). In summary, these data indicate that lipid rafts may serve as signaling platforms driving the formation and growth of the synaptic AChR clusters in the initial phase of NMJ development.

In adult muscle, AChRs are highly concentrated at the synaptic membrane, with a receptor density of \(~10,000–20,000\) AChRs/\(\mu\)m\(^2\), and falling off within a few microns to \(~1–10\)/\(\mu\)m\(^2\) (139). Given this low extrasynaptic AChR concentration, the synaptic AChR cluster is maintained not by trapping of receptors diffusing laterally in the surface membrane, but rather through their focal delivery to the postsynaptic membrane. In principle, the synaptic AChR density is determined by the rates of their insertion into and their removal from the postsynaptic membrane.

Like the accumulation of AChRs described above, this focal insertion of AChRs into the adult synaptic membrane is also regulated by the cytoskeleton but requires, at least in part, a network of microtubules (MTs) associated with the synaptic membrane (208). This network is organized through agrin-induced capturing of MTs at the synaptic membrane and serves to focally transport AChRs to the synaptic membrane (390). MT capturing is mediated by the MT plus-end protein cytoplasmic linker associated protein 2 (CLASP2) interacting with PH-like domain family B member 2 (Phldb2; also called LL5\*), enriched at the synaptic mem-

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**FIGURE 4.** Molecular mechanisms involved in muscle atrophy triggered by denervation. A: action potential activity (red flashes) in innervated muscles suppresses synaptic genes in nonsynaptic myonuclei (white), while their expression is maintained in the fundamental nuclei (black) by agrin-Lrp4-MuSK signaling and the subsequent activation of transcription factors Erm and GABP\(\alpha\)/GABP\(\beta\), which drive expression of AChR-encoding genes (Chrn) and Musk (see text for details). B: denervation results in the immediate loss of electrical activity and strong upregulation of Hdac4 and Hdac5 and downregulation of Hdac9. HDAC4, HDAC5, and HDAC9 induce, via Dach2 and Myog, expression of the E3 ligases Trim63 and Fbxo32 and increase proteasomal degradation. In addition, ACHN levels, including expression of Chrng, are increased in nonsynaptic regions. C: during prolonged denervation, mTORC1 becomes activated and causes an increase in protein synthesis. Feedback inhibition of phosphorylation of Akt/PKB (p-Akt) by S6K enhances expression of Trim63 and Fbxo32 via FoxO transcription factors. mTORC1 also inhibits autophagy via phosphorylating Ulk1. See text for details.
brane by synaptic-specific expression (28, 230) and recruited by synaptic phosphatidylinositol-3,4,5-trisphosphate (PIP3; Ref. 390). Remarkably, synaptic MT capture and focal AChR vesicle delivery also require an intact actin cytoskeleton, consistent with AFD/cofilin-dependent actin reorganization at and AChR delivery to nascent synaptic sites (248). Abolishment of this CLASP2 pathway in vivo lowers synaptic AChR insertion as well as the density and size of synaptic AChR clusters by ~30% each. However, CLASP2-dependent MTs are only one mechanism for focal AChR delivery, as Clasp2−/− mice retain some synaptic MT network (perhaps through its related MT plus-end protein CLASP1), and synaptic AChR density, though lowered, remains sufficient for neuromuscular action potential transmission (390).

The metabolic stability of AChRs is measured by pulse-labeling them irreversibly with α-BTX and monitoring the bound α-BTX over time. In myotubes and in extrasynaptic membrane of denervated muscle, the metabolic stability of the AChRs is low with a half-life of ~1 day. In contrast, AChRs at the mature NMJ have a half-life of 10–14 days. The metabolic stability of the synaptic AChR is not determined by the rate of their internalization alone. Rather, upon their internalization, a fraction of the synaptic AChRs is targeted to degradation, whereas another fraction is recycled to the synaptic membrane (61). Thus the measured metabolic AChR half-life is determined by the net result of the rates of AChR internalization, the fractions of AChRs that are recycled versus degraded after internalization.

As outlined above, AChR anchoring and metabolic stabilization (increase in half-life) of the synaptic AChRs is thought to be achieved by linking them, through the DGC, to the cortical actin cytoskeleton (108). Accordingly, deletion of DGC components impairs metabolic stabilization of synaptic AChRs during synapse maturation. The function of the DGC to stabilize synaptic AChRs is, furthermore, strongly affected by 1) ErbB-dependent phosphorylation of α-dystrobrevin-1 (165, 389) and 2) action potential activity in the muscle as revealed at ectopic endplates denervated early in development, where full metabolic AChR stabilization requires exogenous muscle stimulation (362). Likewise, at mature endplates, where chronic denervation reduces the half-life of synaptic AChRs from ~14 to ~4 days, AChRs are restabilized upon chronic electrical stimulation of the muscle fibers via implanted electrodes (146). This restabilization is mediated by calcium influx through voltage-gated (L-type) calcium channels and is dependent on protein phosphorylation (80). Finally, application of recombinant neural agrin to extrasynaptic regions of the muscle in vivo showed that agrin alone can metabolically stabilize AChRs at ectopic clusters in a dose-dependent fashion (43), a process that is generated by agrin’s action to promote AChR recycling (55).

All treatments affecting synaptic AChR stability examined so far, i.e., removing muscle activity through denervation, blocking of calcium influx, and impairment of DGC function have been found to either shift the fraction of internalized AChRs towards the degradation pathway (calcium, action potentials, deletion of α-dystrobrevin-1 or α-syntrophin) or by selectively accelerating the internalization of the recycled AChR pool (389). However, the molecular mechanisms of how agrin-Lrp4-MuSK signaling assembles the DGC, how the DGC aggregates and stabilizes synaptic AChR clusters, and how muscle activity affects DGC function remain unknown.

VI. MECHANISMS OF MUSCLE WASTING

A. Maintenance of the NMJ and Myasthenias

Many of the molecules involved in the formation of the NMJ have also an essential role in its maintenance. In particular, conditional and/or inducible knockout techniques in mice reveal that NMJs disassemble and become functionally impaired upon loss of agrin, laminin-β2 (375), Lrp4 (23), and MuSK (188, 234). In humans, mutations in genes involved in NMJ function, such as those encoding AChR subunits, ColQ (resulting in the loss of AChE from the NMJ), Na+,1.4, or rapsyn are the most frequent cause of CMS (reviewed in Ref. 124). While this is not surprising based on their necessity in neurotransmission, more recent mapping studies have shown that molecules regulating NMJ development can also cause CMS. These include agrin (200, 283, 323), MuSK (89), Lrp4 (331), and Dok-7 (32). Likewise, sporadic MG, where the majority is caused by autoantibodies to AChRs, can also be caused by antibodies directed against MuSK (193), Lrp4 (189, 342, 479), and agrin (150, 478). Interestingly, there are still many CMS and MS patients where the cause of the disease is not known. Current estimates are that between 30 and 50% of all CMS patients do not have a genetic diagnosis, and in a recent study on 680 patients, this number was even higher than 50% (1). Recent mapping studies and next-generation sequencing approaches have revealed CMS-causing mutations in enzymes involved in glycosylation (34, 101, 394). There is evidence that these mutations can affect glycosylation of AChR subunits and thus impair intracellular trafficking of functional AChRs to the sarcolemma (34). However, it is highly likely that these glycosylation enzymes have additional targets that could participate in the disease phenotype. Thus future research may reveal additional genes involved in the function of the NMJ and may help to further resolve the mechanisms involved in NMJ maintenance.

B. Denervation Causes Muscle Atrophy

NMJ maintenance is important to maintain muscle mass, and thus there is an emerging interest in studying the mo-
lecular mechanisms involved in the loss of muscle mass upon loss of innervation. As highlighted in section VB1, gene expression of synaptic genes in fundamental myonuclei (e.g., Chrm, Musk) is maintained in innervated muscle by sustained activation of agrin-Lrp4-MuSK signaling, while electrical activity suppresses those genes in nonsynaptic myonuclei (Figure 4A). Denervation or malfunctioning of NMJ transmission (e.g., in MuSK-positive myasthenia) results in muscle wasting, which is based on activation or inhibition of specific signaling pathways.

Genome-wide searches for genes whose expression is altered upon denervation led to the identification of a set of transcripts (47, 163, 247). As expression of several of those transcripts is also altered in other muscle atrophy-inducing paradigms, they were termed atrophy-related genes or “atrogenes” (163, 247). Among those atrogenes, tripartite motif containing 63 (Trim63), also called muscle ring-finger 1 (Murf1), and F-box protein 32 (Fbxo32), also called muscle atrophy F-box (MAFbx) or atrogin-1, are two muscle-specific E3 ubiqutin ligases that control substrate specificity of the proteasome. As described below, both E3 ligases have been shown to contribute to the increase in protein degradation and thus the loss of muscle mass during muscle atrophy. Several lines of evidence suggest that this denervation-induced upregulation of Trim63 and Fbxo32 is based on the altered expression of class II histone deacetylases (HDACs), in particular HDAC4 (97, 311, 418) and HDAC5 (311). The increase in the expression of Hdac4 and Hdac5 after denervation triggers a signaling cascade that involves changes in the MAP kinase (93) and the Dach2/myogenin pathways (311, 418; Figure 4B). In addition, denervation causes the downregulation of Hdac9 in nonsynaptic regions (Figure 4B). Forced overexpression of HDAC9 blunts denervation-induced expression of Chrm subunits and of Myog while HDAC9 knockout mice are supersensitive to ACh in response to denervation (297). Thus several HDACs seem to contribute strongly to the changes in gene expression in denervated muscle (Figure 4B).

Changes in class II HDACs and their activation of proteasomal degradation drive muscle atrophy mainly in the initial phase of denervation. If muscle remains denervated for a few days, additional pathways become activated or inhibited, respectively. One of these is the mammalian target of rapamycin complex 1 (mTORC1) pathway, which is a key activator of protein synthesis and inhibitor of autophagy (see sect. VID1; Refs. 246, 468). There is agreement that proteasomal degradation remains increased throughout denervation (Figure 4C). In contrast, the activation state of autophagy during prolonged denervation remains controversial. While it has been proposed that expression of autophagy genes (275, 482) and autophagy flux are increased (285), one recent report indicated that autophagy is inhibited rather than activated upon denervation and that this inhibition is paralleled by the activation of mTORC1 (349). Activation of mTORC1 is not a compensatory response to the activation of proteasomal degradation (and hence protein loss) during denervation, as genetically engineered mice lacking the mTORC1 inhibitor tuberous sclerosis complex 1 (Tsc1) cause accelerated muscle atrophy upon denervation rather than sparing (417). This effect is, at least in part, based on feedback inhibition of S6 kinase (S6K) onto insulin receptor substrate 1 (Irs1; Ref. 433) and thus pronounced inhibition of protein kinase B (PKB, also called Akt; Figure 4C, see also Figure 5A). This, in turn, activates Forkhead box O (FoxO) transcription factors and results in increased expression of Trim63 and Fbxo32 and thus increased protein degradation by the proteasome (Figure 4C). Consistent with this notion, depletion of FoxO1, FoxO3, and FoxO4 protects skeletal muscle from denervation-induced muscle atrophy (417). Finally, there is recent evidence that the mTORC1 inhibitor rapamycin mitigates muscle weight loss and muscle fiber atrophy upon denervation (417), while others have reported an enhancement of muscle atrophy by rapamycin (349).

C. Muscle Atrophy and Muscle Wasting Can Be Triggered by Many Factors

Muscle atrophy can also be triggered by mechanical unloading (e.g., by hindlimb suspension) or immobilization (e.g., casting or as a consequence of spinal cord transection). In these latter conditions, innervation of the muscle remains intact, but muscle activity is decreased. Muscle atrophy can also be observed upon prolonged fasting, a phenomenon that is particularly prominent in mice. Fasting-induced muscle atrophy is based on the requirement to generate free amino acids for protein synthesis in the absence of amino acids supplied by the food. Thus proteins of the skeletal muscle are degraded by autophagy and the proteasomal pathway. Muscle loss (measured as loss of lean mass) can also be the consequence of a primary disease, such as cancer, AIDS, sepsis, congestive heart failure, or chronic obstructive pulmonary disease (COPD), a phenomenon termed cachexia. Although cachexia is secondary to a disease, loss of lean mass is an important prognostic factor in cancer as the higher the extent of weight loss, the shorter the survival time. Finally, muscle loss is also seen as consequence of normal aging. This condition, called sarcopenia, is the main contributor to progressive muscle weakness and frailty and thus contributes substantially to the loss of quality of life in the elderly (364). “Atrogenes” are regulated in a common pattern in all those paradigms (47, 247, 371).

D. Proteostasis and Muscle Homeostasis

Muscle is the largest organ in humans, comprising up to 50% of the total body weight. Because of its high energy demand and its secretory function, muscle is a major con-
with rictor are necessary for the function of the respective complex (see below).

Experiments in yeast and cultured mammalian cells have indicated that mTORC2 affects the organization of the actin cytoskeleton (205, 266, 382). While it is not known in detail how mTORC2 affects the actin cytoskeleton, biochemical evidence shows clearly that mTORC2 controls phosphorylation of the hydrophobic motif of different AGC kinases (128, 149, 203, 383). These include Akt/PKB, protein kinase C (PKC) and the serum- and glucocorticoid-induced kinase 1 (SGK1; FIGURE 5A). AGC kinases are involved in diverse cellular processes including cell growth and actin cytoskeletal regulation and are thought to be deregulated in several disease states including cancer (for recent review on AGC kinases, see Ref. 9).

Germline deletion of Rictor and thus inactivation of mTORC2 in mice causes embryonic death (169, 397). However, tissue-specific deletion of Rictor in several tissues including skeletal muscle results in only minor phenotypes (38, 240). Strikingly, such rictor-deficient skeletal muscle fibers do not show an obvious change in the actin cytoskeleton (38). However, changes in the cytoskeleton and thus in the cell morphology are observed in mice where rictor was depleted in the entire central nervous system or in the forebrain (197, 423). Interestingly, rictor depletion in motor neurons does not affect the NMJ (S. Lin and M. A. Rüegg, unpublished observation). These results indicate that the functions of mTOR in skeletal muscle are mainly carried by mTORC1.

1. Protein synthesis

A) TARGET OF RAPAMYCIN. Among the key regulators of protein synthesis is the target of rapamycin (TOR). TOR was initially identified by genetic screens in yeast that yielded two mutants that were resistant to the growth-inhibitory properties of the immunophilin-immunosuppressant complex consisting of 12 kDa FK506-binding protein 12 (FKBP12) and rapamycin. Rapamycin (also known as Sirolimus) is a macrolide lactone discovered in a soil sample from the Easter Islands as a metabolite of the bacteria Streptomyces hygroscopicus. The two mutants resistant to the FKBP12-rapamycin complex were defective in TOR1 or TOR2 (181). Vertebrates have only one gene that encodes the protein mTOR (for mammalian TOR; also called mechanistic TOR). Rapamycin and its synthetic analogs have been on the market as immunosuppressive drugs for several years; they are in clinical trials also for additional indications including cancer (35).

In yeast, TOR1 and TOR2 associate with particular proteins to form two distinct complexes TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (266). Although there is only one Mtor gene in mammals, the associated components are conserved and also assemble into two distinct protein complexes mTORC1 and mTORC2. Besides mTOR, six additional components constitute mTORC1 and seven components mTORC2 (FIGURE 5A). While some of those components are shared between the two complexes, the proteins regulatory associated protein of mTOR (raptor) and rapamycin-insensitive companion of mTOR (ricktor) are specific for mTORC1 and mTORC2, respectively (FIGURE 5A; Refs. 205, 382). Importantly, raptor and

B) ACTIVATORS OF mTORC1. mTORC1 is a central platform involved in cell growth. Activation of mTORC1 results in an increase in protein synthesis. Stimuli that activate mTORC1 can be nutrient availability, the energy state of a cell, and growth factor signaling. For example, a high ATP/AMP ratio inhibits AMP-activated kinase (AMPK) thereby releasing its inhibition on mTORC1 (FIGURE 5B).

Amino acids, in particular branched-chain amino acids (e.g., leucine or arginine), activate the lysosome-attached vesicular ATPase (v-ATPase) and acidify the lysosomes. The v-ATPase controls Regulator and the guanine nucleo-

FIGURE 5. mTORC1 and mTORC2 signaling in skeletal muscle. A: cross talks between mTORC1 and mTORC2 complexes in the maintenance of muscle homeostasis. Activation of the mTORC1 complex in skeletal muscle requires 1) amino acid-dependent activation of the Rag-GTPase, docked at the lysosomal surface by the Regulator complex, and 2) the insulin/IGF1/growth factor-dependent activation of the PI3K-Akt/PKB pathway. Active Akt/PKB induces phosphorylation (step 1) and dissociation (step 2) of the TSC1-TSC2 complex from the lysosomal Rheb GTPase. Rheb-GTP recruits and activates mTORC1 at the lysosomal surface. Activation of Akt/PKB or S6K1, which involves mTORC2, also prevents the nuclear accumulation of FoxO transcription factors, thereby preventing expression of the "atrogenes." Importantly, the mTORC1 target S6K1 initiates a feedback loop and inhibits Irs1, thereby dampening activation of Akt/PKB and preventing hyperactivation of mTORC1. B: mTORC1 orchestrates protein synthesis and degradation. The phosphorylation of 4EBP by mTORC1 on multiple sites (step 1) causes the dissociation of 4E-BP from eIF4E (step 2) and induces the assembly of the 43S preinitiation complex (PIC) and the initiation of 5TOP mRNA (5' terminal oligopyrimidine) translation. On the other hand, mTORC1-dependent phosphorylation of Ulk1 prevents the induction of autophagy. For further details see text.
tide loading of the two ras-related GTPases (Rags) RagA and RagB (21). RagA/B and RagC/D heterodimers directly bind to raptor, if they are in the proper nucleotide-bound state (22; **FIGURE 5A**). This recruits mTORC1 to lysosomes and causes its activation by the small GTPase ras homolog enriched in brain (Rheb), which also resides at the lysosomal surface (**FIGURE 5A**). Recent observations have, in addition, shown that TSC1 and TSC2 also participate in the activation/inhibition of mTORC1 by amino acids. TSC2 (also known as tuberin) forms a complex with TSC1 (also known as hamartin). Phosphorylation of TSC2 by Akt/PKB (see below) downregulates the GTPase-activating protein (GAP) activity of the TSC1/TSC2 complex and thereby activates Rheb (**FIGURE 5A**). In the absence of amino acids, TSC2 is attached to the lysosome and inhibits Rheb (114). Growth factor stimulation in the presence of amino acids dissociates TSC2 from the lysosome and releases inhibition of Rheb, which, in turn, leads to activation of mTORC1 (299). The release of TSC2 from the lysosome involves its phosphorylation by Akt/PKB (299). These findings raise the possibility that subcellular localization of TSC2 is a general mechanism for regulating the TSC1/TSC2 complex by amino acids and growth factors, and they suggest a cross-talk between amino acid availability and growth factor signaling.

C) TARGETS OF mTORC1. The two main downstream signaling components of mTORC1 are S6K and eukaryotic translation initiation factor 4E (eIF4E) binding protein (4EBP), which together control protein synthesis (**FIGURE 5B**). Phosphorylation of 4EBP induces its release from eIF4E, which enables assembly of the 40S ribosome with the ternary complex of eIF2, M7-tRNA, and GTP at the 5'-cap of mRNA and thus the formation of the preinitiation complex to initiate cap-dependent translation. Phosphorylation of S6K also participates in the assembly of the preinitiation complex (194) by activation of eIF2, and it phosphorylates S6 on several sites (**FIGURE 5B**). Taken together, activation of mTORC1 controls diverse steps in protein synthesis by its effect on 4EBP and S6K (**FIGURE 5B**).

2. The insulin/IGF1-PI3K-Akt/PKB pathway

Another important input that results in the activation of mTORC1 is growth factors. In contrast to simple organisms, growth factor availability is the main determinant of organ size in mammals. The best-characterized pathway that affects mTORC1 activation is initiated by insulin/insulin-like growth factor 1 (IGF1). Specifically, binding of insulin/IGF1 induces autophosphorylation of its receptor, which creates docking sites for Irs1 and other scaffolding proteins (**FIGURE 5A**). These scaffolding proteins, in turn, become phosphorylated by the receptor and activate the p85 regulatory subunit of class I phosphoinositide 3-kinase (PI3K). Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) into PIP3 to recruit and activate Akt/PKB via 3-phosphoinositide-dependent protein kinase 1 (PDK1) and the participation of mTORC2 (**FIGURE 5A**). Activation of Akt/PKB causes phosphorylation of TSC2 upon dissociation of TSC2 from the lysosome (see above), which then results in the activation of mTORC1 via Rheb (see above).

Akt/PKB has many additional downstream targets (see overview in Ref. 182). One such target that also plays a role in skeletal muscle atrophy is glycogen synthase kinase-3 (GSK-3), which becomes phosphorylated by Akt/PKB. Interestingly, GSK-3 affects multiple pathways such as glycogen synthesis, Wnt/β-catenin signaling, and protein synthesis, which all have been shown to play a role in skeletal muscle (157, 368). Recent evidence also indicates that GSK-3 directly phosphorylates neighbor of BRCA gene 1 (NBR1) to prevent aggregation of ubiquitinated proteins and to enhance their autophagosomal degradation (324). In addition to the above activities of Akt/PKB, phosphorylation and thereby inhibition of FoxO transcription factors are most significant for the control of muscle mass (**FIGURE 5A**). Specifically, phosphorylation of FoxOs exports them from the myonuclei back to the cytoplasm and thus prevents the expression of the FoxO targets (379, 409). Among those targets in skeletal muscle are the “atrogenes,” which include the E3 ligases Trim63 and Fbox32 and genes involved in autophagy (**FIGURE 5A**; see details below). Another upstream kinase that phosphorylates FoxOs is SGK1. Like Akt/PKB, SGK1 inhibits FoxO3 by phosphorylation (63). Interestingly, SGK1, but not Akt/PKB, is activated during hibernation in squirrels to protect muscles from atrophy (5). Thus activation of Akt/PKB increases protein synthesis via mTORC1-dependent activation of translation and inhibits protein degradation via FoxO-dependent inhibition of proteasomes (**FIGURE 5**). This dual function of Akt/PKB probably explains the profound effect of the over-expression of a constitutively active form of this kinase on muscle size (204, 244).

3. Protein degradation

The two most important protein degradation pathways during muscle atrophy are the autophagy/lysosomal pathway and the ubiquitin-proteasome system (UPS). We will not discuss caspases, whose activation is required to trigger apoptosis and the calcium-activated calpains, which participate in various calcium-mediated intracellular signal transduction pathways (reviewed in Refs. 161, 265).

A) AUTOPHAGY. mTORC1 is also a main regulator of autophagy (“self-eating”), a catabolic process that degrades cellular components via the lysosomal pathway. Autophagy can be grouped into macroautophagy (usually referred to as autophagy), microautophagy, and chaperone-mediated autophagy (307). Autophagy is active at low levels in all cells and is highly induced by situations of stress where it can act as a survival mechanism. Dysregulation of autophagy has been implicated in many diseases (see review in Ref. 91).
For example, excessive autophagy is thought to contribute to neurodegenerative diseases including Alzheimer’s disease (470), whereas autophagy is inhibited in Huntington’s disease (326). Similarly, autophagy flux is perturbed in mouse models for different muscular dystrophies (79, 168).

Autophagy is a highly dynamic process that is controlled in many tissues by mTORC1 via its inhibitory function on unc-51-like kinase 1 (ULK1). ULK1 is part of the large ULK1 complex (FIGURE 5A; reviewed in Ref. 245). The inhibitory function of mTORC1 is mediated by phosphorylation of Ser757 in ULK1, which prevents activation of the ULK1 complex by AMPK (225). Activation of the ULK1 complex and, equally important, activation of a protein complex containing the core components Beclin1 and vacuolar protein sorting 34 (Vps34), called Beclin 1/Vps43 complex (148, 366), results in the formation of phagophores (FIGURE 5B). This nucleation step is followed by the recruitment of microtubule-associated protein 1 light chain 3 (MAP1LC3), more commonly referred to LC3, and of target proteins, which in turn are recruited via adaptors proteins such as sequestosome 1 (SQSTM1/p62). Phosphatidylethanolamine (PE) is conjugated to LC3 at its COOH terminus (LC3-PE) to associate with the core components of the autophagosomes, intermediate structures that are characterized by their double membrane (FIGURE 5B). With the assistance of lysosome-associated membrane protein 2 (LAMP2), the autophagosomes then fuse with lysosomes, resulting in the formation of autolysosomes where the proteins are degraded and recycled (FIGURE 5B). The tight control of the autophagy process by mTORC1 is a mechanism to couple sensing of amino acids to autophagy. In the presence of amino acids (i.e., fed state; mTORC1 active), autophagy is inhibited, while in the absence of amino acids (i.e., starved state; mTORC1 inhibited) autophagy is activated. Thus activation of autophagy by the inhibition of mTORC1 allows the release of free amino acids to maintain a basal level of protein synthesis (307).

In contrast to many tissues, autophagy in skeletal muscle has been proposed to be mainly controlled by Fox03 signaling (FIGURE 5A) and not by mTORC1 (275, 482). This view is based on the inability of the mTORC1 inhibitor rapamycin to increase autophagy flux in skeletal muscle (275). For example, although rapamycin was shown to increase autophagy flux in the heart, autophagy in skeletal muscle was not affected (92, 350). However, the failure of rapamycin to affect autophagy is more likely based on its inability to sufficiently penetrate skeletal muscle, as sustained activation of mTORC1 by the conditional deletion of Tsc1 in skeletal muscle results in a complete blockade of autophagy induction (83). As a consequence of this inhibition, mutant mice suffer from a late-onset myopathy (83), which is reminiscent of that caused by the skeletal muscle-specific deletion of autophagy-related protein 7 (Atg7; Ref. 285), one of the “core” Atg proteins (307). Despite the now strong evidence that induction of autophagy in skeletal muscle is regulated by mTORC1, long-term maintenance of a proper autophagy flux requires increased expression of autophagy genes. In skeletal muscle, FoxOs have been shown to affect transcription of several autophagy genes including Map1lc3b, Gabarapl1, and Bnip3. Some of those genes are direct targets of FoxO3 as demonstrated by chromatin immunoprecipitation (275, 482). Thus the role of FoxO3 is mainly in the regulation of protein degradation by transactivation of genes involved in the UPS and the autophagosomal/lysosomal pathway (FIGURE 5A). Accumulating evidence suggests the existence of a complex crosstalk between these two processes. It has recently been reported that upon denervation, accumulation of Trim63 at the NMJ regulates both the recycling of AChRs as well as the trafficking of autophagic vacuoles (367).

**B) THE UPS.** While protein synthesis is largely under the control of mTORC1, muscle atrophy involves primarily the activation of the UPS, which is also called UPP for ubiquitin proteasome pathway (358, 372). Pioneering work has established that muscle atrophy induced by different paradigms involves increased transcription of components of the proteasome and a two- to threefold increase in proteolytic activity (292).

The ATP-dependent proteasome activity largely depends on protein ubiquitination. The proteolytic activity of this oligomeric protease resides in the inner, barrel-shaped structure of the 20S core particle, which assembles with the 19S regulatory particle to the 26S proteasome (315), where the substrate proteins are hydrolyzed (FIGURE 6A). The ubiquitination process sequentially involves 1) activation of the free ubiquitin moiety by a unique and ubiquitously expressed ubiquitin-activating enzyme E1 (UBE1); 2) conjugation of activated ubiquitin to the ubiquitin carrier protein (Ubc) or E2 protein; and 3) recognition of the substrate and transfer of the activated ubiquitin to the substrate by a ubiquitin ligase or E3(ligase). The addition of a polyubiquitin chain to the ε-amino group of the lysine of the substrate determines the fate of the target protein (FIGURE 6A; for review, see Ref. 186). According to the “ubiquitin code,” the ubiquitin monomers can be polymerized at seven possible lysines (Lys 11, 27, 29, 33, 48, or 63), and the side chain of lysine 48 is responsible for the targeting of the protein substrate to the 26S proteasome (187, 474). Other modifications such as polyubiquitination of lysine 63 are thought to modify activity, trafficking, and/or localization of the modified substrate.

Although the E2 enzymes may display some ubiquitin conjugation specificity, the E3 ligases are thought to control specificity (FIGURE 6A; Refs. 186, 424). More than 600 E3-like genes are found in the human genome that belong to two major classes; the homologous to E6-AP COOH-ter-
A. **E3 ligase**

<table>
<thead>
<tr>
<th>HECT</th>
<th>RING finger</th>
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</thead>
<tbody>
<tr>
<td>Nedd4-1</td>
<td>CRL2, 3, 4A, 4B, 5, 7</td>
</tr>
<tr>
<td>Substrate</td>
<td>Multi-subunits</td>
</tr>
<tr>
<td>CUL7 (or CUL2, 3, 4A, 4B, 5)</td>
<td>CRL1/SCF</td>
</tr>
<tr>
<td>Fbxo30, Fbxo40</td>
<td>Single-subunit</td>
</tr>
<tr>
<td>Nedd8</td>
<td>Trim3</td>
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<tr>
<td>RING finger</td>
<td>cbl-c</td>
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<tr>
<td>N8</td>
<td>RING finger</td>
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B. **Mitochondrion cycle**

- Fusion
- Fission
- Mitophagy
- ER
- Myogenic program
- Protein synthesis
- mTORC1
- mTORC2
- PI3K
- S6K1
- Rictor
- Raptor
- mRNAs
- Myostatin
- IGF1
- IGF1R
- TNFα
- Smad
- NFκB
- HDAC4
- PDK1
- Akt
- FoxO
- MyoD
- Myogenin
- CRL1/SCF
- Trim63
- Fbxo25
- CUL1
- Rbx1
- Skp1
- Nedd8
- N-end rule protein
- F-box protein
- CUL
- RING finger
- E2
- E3 ligase
- Thioester bond
- Ubiquitin
- ATP
- ADP
- Opening
- Unfolding
- Cleavage
- Release
- Peptides
- Amino acids
- Homotypic or mixed Ub chain
- Polyubiquitinated substrate
- Substrate-binding specificity (motif-PTM-degron) and ubiquitin chain transfer

C. **Substrate-binding specificity**

- α-actinin
- Actin
- Myosin
- Z disk
- M-band
- Sarcomere
- Nucleus
- Mitochondrion
- Cytosol
- Plasma membrane
- IGF1-R
- PI3K
- PD1
- Irs1
- p110,p85
- IGF1 insulin
minal (HECT) domain-containing enzymes that transfer ubiquitin from the E2-loaded enzyme to the substrate via a specific E3-ubiquitin thioester intermediate (196, 198) and the cullin/RING-finger ubiquitin ligases (CRL), which are mainly nonenzymatic recognition factors that scaffold the substrate in close proximity [FIGURE 6B]. The functionality of such complexes relies on the ability of the cullins to link the polyubiquitin-loaded E2 with the specific substrate to be degraded (483). This assembly requires the binding of a RING-finger adapter Ring box-1 (Rbx 1) at the COOH-terminal end of cullin, which then recruits the E2 enzyme loaded with ubiquitin [FIGURE 6B]. At the NH2 terminus, cullin binds to the substrate-specificity module composed of the S-phase kinase adaptor-1 (Skp1) protein and the Fbox protein to form the Skp1-Cul1-Fbox complex or SCF [FIGURE 6B]. Modification of the cullin subunit by NEDDylation has been proposed to be important for the activation of the SCF complex. NEDDylation is the posttranslational modification where a ubiquitin-like protein, the neuronal precursor cell expressed, developmentally downregulated protein 8 (Nedd8) is conjugated to the lysine of the substrate (Lys 720 in Cul1). At the protein level, more than 1,300 different E2/E3-RING ligase complexes are found in mammals, which in combination with the 69 Fbox proteins identified so far, are thought to determine substrate specificity (33, 233, 279).

The E3 ligases so far identified in skeletal muscle belong to the RING-finger domain family except neural precursor cell expressed developmentally downregulated protein 4 isoform 1 (Nedd4-1), whose expression is induced during muscle atrophy (29). Although the detailed mechanisms are not known, muscle-specific Nedd4-1 knockout mice display some protection of fast-twitch fibers to denervation-induced atrophy (318). The protein of the RING-finger domain family that is induced by denervation the most is Fbxo32. Fbxo32 transcripts are also increased during fasting, hindlimb suspension, immobilization, oxidative stress, or sepsis (47, 163, 372, 409), which assigns Fbxo32 to the group of the “atrogenes” (sect. VIB). It is well established that the FoxO transcription factors, via the insulin/IGF1-PI3K-Akt/PKB pathway, are important regulators of the “atrogenes” [FIGURE 6C]. However, other pathways that not necessarily make use of FoxOs have also been implicated in the regulation of “atrogenes” and in the control of muscle atrophy. Examples are the p38/MAP kinase (255) and the NF-κB pathways (74, 409).

Fbxo32 is an atypical Fbox protein because the Fbox motif is located in the COOH-terminal part and because it lacks the common substrate binding domain (SBD) found in other Fbox proteins. Fbxo32 forms a multicomponent E3 ligase of the CRL1 or SCF subfamily of the RING finger E3 ligases [FIGURE 6B]. In skeletal muscle, Fbxo32 has been shown to target the degradation of the myogenic determinant (MyoD; Ref. 425), a key transcription factor driving myoblast differentiation during myogenesis and regeneration, and the regulatory subunit f of the eukaryotic initiation factor 3 (eIF3f) of translation (106, 107, 243; [FIGURE 6C]. As eIF3f also participates in the activation of S6k by mTORC1 to form the preinitiation complex of translation (see also [FIGURE 5B], Fbxo32 inhibits protein synthesis (14, 206, 247). Knockout animals for Fbxo32 are fertile and display no obvious phenotype compared with their littermates. However, when subjected to denervation or starvation, their muscles are partially resistant to atrophy (56% sparing), suggesting a role for Fbxo32 in regulating muscle size (47). However, denervated muscle from Fbxo32-deficient mice becomes severely myopathic 28 days postdenervation (instead of 14 days as in Ref. 47), suggesting that Fbxo32 is necessary for maintaining muscle integrity upon denervation (162).

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**FIGURE 6.** UPS function and regulation in skeletal muscle. A: principles of ubiquitin-mediated protein degradation. Protein ubiquitination involves the sequential activation and conjugation of ubiquitin monomers by E1 and E2 enzymes. This ATP-consuming process uses the COOH-terminal glycine residue of ubiquitin and loads it on the cysteine residues of the E2-conjugating enzyme via a thioester bond (exchanged upon trans-esterification with E1-Ub). E3 ligases further regulate the ultimate step of ubiquitination, ensuring both the recognition of the substrate as well as the transfer of the ubiquitin from E2 enzymes to one or several lysine residue of the target substrate (5). The polymerization of the ubiquitin chain involves the combination of ubiquitin monomers through linkage via one of the 7 possible lysine binding sites of ubiquitin (Lys 11, 27, 29, 33, 48 and 63). The resulting "ubiquitin code" determines the fate of the substrate. Degradation of the ubiquitinated protein in the 26S proteasome requires the release of the ubiquitin chain by deubiquitinating enzymes (DUBs) and activates the opening of the proteasome lid. The proteolytic activity of the proteasome is located in the β-subunits (as showed by colored stars corresponding to chymotrypsin-like, trypsin-like, and caspase-like activity) and generates short polypeptides (6–8 amino acids long) that are further processed by cellular peptidases and contribute to the replenishment of the amino acid pool in a cell. B: identity and structure of the muscle E3 ligases involved in muscle wasting. The RING finger E3 ligases involved in muscle wasting are functional complexes, called cullin/RING-finger ubiquitin ligases (CRL), that lack enzymatic activity (except for the member of the HECT family, Nedd4-1) and function as scaffolding platform that specifically load their substrate in close proximity with the E2-ub intermediates. Fbox proteins are essential to build a functional E3 ligase and their diversity, in association with the different members of the cullin family, accounts for the substrate specificity of the CRL. NEDDylation (NB stands for Nedd8) of the members of the cullin family is required to activate the E3 ligase complex. C: proposed model for E3 ligase-mediated muscle breakdown. Activation, during muscle wasting, of catabolic pathways leads to the transcriptional upregulation of several components of the UPS, such as the E3 ligases Fbxo32/29/40, Mul1, and Trim63. Although the molecular mechanisms responsible for the activation and the specificity of the corresponding newly assembled E3 ligase complexes are largely unknown, they control the targeted degradation of substrates involved in transcription, translation initiation, and growth signaling via the formation of functional CRL complexes. In addition, structural components (sarcomere) and metabolic organelles (mitochondria) are degraded via single subunit ring finger protein Trim63 and Mul1, respectively. For details see text.
In addition to Fbox32, Fbxo29 (also called F-box and WD repeat domain containing 8; FBXW8) and Fbxo40 also affect mTORC1 signaling (Figure 5C). Fbxo29 has been proposed to establish a feedback regulatory loop between the mTOR and Akt/PKB pathway by targeting the degradation of the phosphorylated form of Irs1 (228), and Fbxo40 seems to target the degradation of activated Irs1 in muscle cells (395, 473). Another member of the Fbxo family involved in muscle atrophy is Fbxo30, also called muscle ubiquitin ligase of the SCF complex in atrophy 1 (MUSA1). It becomes activated in the absence of the prohypertrophic, transforming growth factor-β family member bone morphogenetic protein (BMP) (Figure 6C; Refs. 384, 457).

Nonconventional single RING-finger E3 ligases are also involved in muscle atrophy. One of those is Trim63 (Figure 6B), which was coidentified with Fbxo32 to be upregulated in muscle atrophy-inducing paradigms (47, 163). Trim63 localizes to the M line of the sarcomere, and its function in muscle atrophy (Figure 6C) has been linked to its capacity to target degradation of key structural components, such as myosin heavy chain (MyHC), the myosin light chains MyLC-1 and MyLC-2, and the myosin binding protein-C (MyBP-C; Refs. 95, 96). Trim63 has also been implicated in muscle metabolism by targeting the muscle-type creatine kinase for degradation (235). The gastrocnemius muscle of Trim63 knockout mice shows some resistance to atrophy induced by denervation or starvation (36% sparing). The muscle-sparing effect in Trim63 knockout mice seems, at least in part, also to be attributed by the absence of HDAC4 induction (147). Trim63 shares more than 60% sequence identity with its related isoforms Trim55/MuRF2 and Trim54/MuRF3 (86). Despite their conserved function in the UPS, only Trim63 has been reported to be upregulated in response to denervation and starvation (163). Trim63 thus appears as an attractive candidate for pharmacological intervention in response to denervation. A first compound has recently been shown to inhibit Trim63 in a dose-dependent manner in C2C12 myoblasts (123).

Another RING-finger protein, casitas B-lineage lymphoma proto-oncogene (cbl-c), has also been reported to be upregulated in disused muscle (330). Cbl-c has been proposed to mediate the degradation of Irs1 when phosphorylated at tyrosine residue 608 in unloaded muscles (319). Consistently, unloaded Cbl-c-deficient muscles display high levels of phosphorylated FoxO3, suggesting that the restoration of Irs1 signaling activates Akt/PKB (319). More recently, the RING-finger domain ligase mitochondrial E3 ubiquitin protein ligase 1 (Mul1) has been identified as a new FoxO3/1-regulated gene that targets the degradation of mitofusin 2 (Mfn2). Mul1 activates mitochondrial fragmentation and loss during atrophy via a process called mitophagy (267). Importantly, this process seems to be regulated in a fiber type-dependent fashion.

Consistent with the concept that inhibition of UPS affects muscle wasting, proteasome inhibitors, such as MG132, prevent muscle atrophy in rats and ameliorate the disease in mdx mice, the mouse model of Duchenne muscular dystrophy (50, 131, 421). More recently, the new peptidomimetic inhibitors of the 20 core particle peptidases, such as Bortezomib, have been shown in vivo to be protective against myofibrillar breakdown and are currently tested in clinical trials (31, 213). The identification of specific E3 ligases being involved in muscle wasting has raised the hope to find more selective drugs that avoid the deleterious side effects of long-term inhibition of the proteasome activity (258). To this end, several questions remain to be answered. For example, which signals activate Fboxo32 and Trim63; what is the nature of the E2 complex associated with these E3 ligases; and which proteins are selectively targeted by these complexes during denervation-induced muscle wasting?

VII. LOSS OF MUSCLE MASS IN AGING (SARCOPENIA)

In previous sections, the development and function of the NMJ including the molecular mechanisms involved have been discussed. As perturbation of NMJ function has a strong impact on muscle metabolism and performance, and ultimately on muscle size, we then described the most important molecular pathways that are involved in the control of muscle size. In this final section, we discuss possible mechanisms involved in the loss of muscle mass in aging (called sarcopenia). Although the mechanisms underlying sarcopenia are still poorly understood, there is evidence that the function and structure of NMJs are altered during aging and that the mechanisms involved in the regulation of muscle mass discussed in the previous section affect NMJs. This section will provide insights into those mechanisms and will discuss of how future research can help to better understand them.

A. Sarcopenia Is a Multifactorial Syndrome of Old Age

The term sarcopenia (360) was coined in 1988 and means loss of flesh or muscle [from the Greek words of “σαρκός” (flesh) and “πενῶς” (deficiency)]. While originally defined as loss of lean body mass, it has subsequently come to include functional parameters. According to recently established guidelines (104), sarcopenic patients need to fulfill two criteria. The first criterion is the loss of muscle mass, measured as “skeletal mass index” (SMI), which represents the muscle mass in kilograms divided by the square of the body height in meters (kg/m²). One of the challenges with this parameter is the fact that muscle mass can be measured with different methods. For example, if lean body mass is measured by dual-energy X-ray absorptiometry (DXA) scan, the mean SMI of young men is 8.6 ± 1.1 (SD) kg/m².
and of young women $7.3 \pm 0.9 \text{ kg/m}^2$ (30). Pathological loss of muscle mass was defined as being more than two standard deviations below the mean SMI in young adults (104). The second criterion for sarcopenia is poor physical performance, such as low gait speed (e.g., $<0.8 \text{ m/s}$ measured in a 6-min walk test) or poor grip strength. While the correlation between impaired physical performance and low muscle mass is weak (as there are many reasons for poor physical performance), the correlation between low muscle mass and impaired physical performance is stronger (249). Consistent with sarcopenia having a major impact on function, it correlates highly with the incidence of falls (419).

The definition of clear-cut criteria for sarcopenia is important for its recognition as a syndrome, and it is essential for calculating the prevalence of the syndrome and for estimating the resulting health care costs. So far, most epidemiological studies did not include functional parameters (e.g., Ref. 30), which explains the rather large difference in the observed prevalence between studies (see Ref. 109). According to a recent study in more than 700 people from northern Italy, which used both SMI and physical performance as inclusion criteria, 31.6% of women and 17.4% of men aged 80 years or older were sarcopenic, and the prevalence of sarcopenia increased steeply with age (442).

Sarcopenic muscle is characterized by muscle fiber atrophy (i.e., smaller average fiber diameter) and an increase in fiber size heterogeneity; reduction of the total number of muscle fibers; selective loss of fast-twitch (type II) and preponderance of slow-twitch (type I) fibers; presence of “mixed” fibers (expressing both slow and fast isoforms of myosin heavy chain); fiber type grouping (indicative of reinnervation by nonaffected motor neurons); presence of central myonuclei; and infiltration of nonmuscle cells (e.g., adipocytes and connective tissue cells). In addition, old muscle regenerates poorly upon injury due to decreased regenerative capacity of aged satellite cells (see review by Ref. 53). This multitude of phenotypes indicates that most likely several factors contribute to sarcopenia. Specific factors discussed are hormonal dysregulation (i.e., changes in testosterone, estrogen, growth hormone, IGF1) and muscle-specific changes in the anabolic or catabolic pathways, in mitochondrial function, and in excitation-contraction coupling (105, 229). All these intrinsic alterations in combination with changes in daily activity and in nutritional habits (e.g., resulting in sarcopenic obesity; Ref. 363) may contribute to the loss of muscle. As there is no particular treatment available for sarcopenia at the moment, the most successful “therapeutic” interventions to prevent or slow-down sarcopenia are lifestyle based. They include changes in caloric intake and in the composition of food, combined with an exercise regime of resistance and aerobic training (287).

### B. Changes at the NMJ During Aging

#### 1. Human studies

It is not clear whether changes of the NMJ in sarcopenic muscle are primarily triggered by changes in the motor neuron or in the muscle fiber. Indications for a presynaptic involvement are muscle atrophy and fiber type grouping, two clinical manifestations that are also hallmarks of ALS. Indications for a postsynaptic origin are the increase in the relative number of central myonuclei, characteristic of muscle fiber degeneration/regeneration, and alterations in excitation-contraction coupling, whereas changes in muscle metabolism and mitochondrial function could be either of pre- or postsynaptic origin. As both cells interact with each other at the NMJ, the numerous structural alterations at NMJs observed in the course of aging are not surprising (see below). What is not known, however, is whether these changes reflect NMJ dysfunction as a primary cause of sarcopenia or whether impaired NMJ function is secondary to age-related motor neuron and/or muscle fiber dysfunction.

It is important to note that these questions have not been unequivocally resolved so far. For example, structural alterations at the NMJ correlate with age (329, 460), but a correlation neither with sarcopenic muscle nor with NMJ function has been established. Specifically, age-dependent morphological changes include an increase in the length, the branching, and the overall area of the postsynaptic membrane and increased perijunctional AChR labeling near old NMJs (329). Electron microscopy revealed the presence of degenerating synaptic folds that were often not opposed by a presynaptic nerve terminal. Finally, terminal Schwann cells tended to invade the synaptic clefts more frequently (460). These studies thus indicate that the number of “pathological” NMJs increases with age. However, it still needs to be established whether these changes are more frequent in sarcopenic compared with normally ageing muscle.

Age dependence of NMJ function has been examined electrophysiologically by single fiber electromyography (SFEMG), a technique to record transmission of action potentials from single muscle fibers in response to nerve stimulation or voluntary activation of skeletal muscles. SFEMG enables analysis of the efficacy of neuromuscular transmission (216). The values for jitter, i.e., the variability in transmission time between the point at which the axon is stimulated and the position along the muscle fiber at which the action potentials are recorded, can be used as an estimate of the safety factor of neuromuscular transmission (216). An increase in jitter indicates a lower safety factor. SFEMG also allows the measurement of fiber density, which is thought to represent the size of the motor units. Meta-analysis of the jitter values and the fiber density in different muscles has shown that both values increase with age (19, 59). Again, however, SFEMG has not been measured specifically in sarcopenic patients.
2. Rodent studies

As humans are not amenable to large-scale and detailed studies of the NMJ, most molecular and interventional studies have examined NMJs in aged rodents. Like humans, aged mice and rats (older than 24 mo) show loss of skeletal muscle mass. These changes are very consistent among individuals, but the extent of muscle loss may differ between rodent strains and may also depend on the holding conditions. Close examination of the NMJs in aging mice and rats has revealed that they undergo marked morphological remodeling. The phenomena include a high percentage of fragmentation and a substantial degree of partial and complete denervation concomitant with the appearance of mixed fibers (88, 254, 365, 435). Moreover, postsynaptic AChR staining becomes fainter (88, 254, 435). Functional changes include a marked drop (by 45%) of the nerve-evoked peak tetanic force in the EDL muscle of 24-mo-old rats compared with 6-mo-old controls (201). Presynaptic changes include the thinning and swelling of motor axons. Interestingly, the extent and onset of the structural changes differ greatly between muscles, and the differential response of muscle correlates well with the extent of NMJ changes observed in a mouse model of ALS (436). Whereas it is thought that the synaptic changes are gradual and slowly progressing over time, repeated vital imaging has revealed that changes can occur rapidly (254). These sudden changes correlate with the local degeneration and regeneration of the muscle fibers. Thus one mechanism that may lead to the fragmentation of the NMJ during aging might be local degeneration/regeneration of those muscle fiber segments underlying the NMJ (254), as is also observed in the mdx mouse, a model for Duchenne muscular dystrophy (273).

Curiously, the frequency but not the amplitude of mEPPs is decreased in 29- to 33-mo-old C57/Bl6 mice (20) and in 28-mo-old C57/Bl6 mice (129). The amplitudes of EPPs were in both cases even increased, and few, if any, electrophysiological indications of denervation were observed (20, 129). Given that muscle fibers become smaller in aged mice, their input resistance will increase, which may, in part, explain the increase in EPP amplitude. A change in mEPP frequency and not amplitude has also been described in a mouse model of a severe MuSK-based CMS (90), although in this case, the muscle force upon nerve stimulation was much lower than in control animals. Thus there is little evidence for a functional impairment of neuromuscular transmission in old mice despite the strong fragmentation of the NMJ. This is in stark contrast to data from human and rats, where neuromuscular transmission at high age is clearly diminished (see above).

Gene expression analyses in sarcopenic rats have revealed prominent signs of denervation. Molecular signatures include upregulation of Chbna1 (α subunit of AChRs), Musk, and Lrp4 (25, 201). Moreover, interventions that have been shown to slow down sarcopenia, such as caloric restriction and exercise, also normalize the structure of aged NMJs (88, 129, 435). Finally, conditional deletion of agrin in adult motor neurons (375) or removal of agrin from the NMJ by overexpression of the agrin-selective protease neurontypsin in motor neurons (49) results in the fragmentation and denervation of the NMJ reminiscent of the changes observed at aged synapses. Interestingly, muscles in these transgenic mice show many of the general features of sarcopenia already at a young age (73). In summary, the findings described above and the fact that impairment of neuromuscular transmission (as observed in CMS or sporadic MG) is usually associated with structural changes of the NMJ suggest that impairment of synaptic function may contribute to sarcopenia. However, it is not established whether changes of the NMJ are the cause or the consequence of sarcopenia. Thus unequivocal demonstration of NMJ involvement would require a more systematic examination of NMJ function and structure at clearly defined disease states. The assessment of functional innervation of the entire muscle could for example be done by comparing the muscle force generated in response to stimulation through the nerve with that to direct electrical stimulation of the muscle as has been done in a mouse model of a severe congenital muscular dystrophy (170). This would allow distinguishing between impairment of neuromuscular transmission and general weakness of the muscle.

C. Mechanisms Involved in Sarcopenia

As mentioned above, many different factors have been implicated in the triggering of sarcopenia, and it is likely that several of those factors also affect the NMJ. Similarly, the fact that muscle mass is lost in sarcopenia has led to the concept that mechanisms involved in acute muscle atrophy (for example those induced by denervation) are also causative to sarcopenia. Whether this is indeed the case is not resolved.

D. Connection Between Pathways That Affect Lifespan and Sarcopenia

As sarcopenia is an age-dependent syndrome, many studies have linked longevity and sarcopenia. One of the best-characterized experimental paradigms that prolong lifespan is caloric or dietary restriction (CR or DR). In CR, daily caloric intake is limited to ~60% of that under ad libitum conditions without causing malnutrition. This paradigm has been shown to prolong lifespan in many species (343), although recent work has questioned the universality of the phenomenon. For example, in some mouse strains, CR shortens lifespan (257), and recent long-term experiments have not been able to repeat the initial report (98) that CR significantly increases lifespan in rhesus monkeys (289). While these controversial results are disturbing at first sight, they may in fact argue that multiple pathways are involved...
in mediating the effect of CR on lifespan. Thus the observed heterogeneity between strains and between experimental paradigms could in fact be used to unravel the genetic basis for the observed lifespan extension (see recent review in Ref. 256).

Attempts to unravel the genetics of CR have resulted in the identification of several candidate pathways. These include the sirtuins, which are a class of nicotinamide adenine dinucleotide (NAD)$^+$-dependent enzymes (171). Most of the sirtuins have deacetylase activity. Cellular aging is accompanied by changes in mitochondrial function, and CR has been shown to positively affect mitochondrial function and to increase deacetylation. As SIRT3 is required for many of the deacetylation processes activated in mitochondria (180), a role of sirtuins in cellular aging has been suggested. Indeed, overexpression of sirtuins and thus increased deacetylation promotes longevity in several species (219, 426). However, others have not been able to reproduce this effect (71). Thus, similar to CR, differences in the experimental set-up, in the genetic background of the animals, or in the degree of overexpression in the different tissues may affect the outcome.

SIRT1 also deacetylates peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC1α or PPARGC1a), a transcriptional coactivator that has been shown to be a key regulator of mitochondrial biogenesis (359). Endurance exercise induces expression of PPARGC1a in human skeletal muscle (344), and transgenic overexpression of PGC1α in skeletal muscle of mice increases the number of mitochondria and the oxidative properties (259). Interestingly, endurance training in mice also reduces the fragmentation of the NMJ in aged mice (88, 435), and overexpression of PGC1α in skeletal muscle improves the morphology of the NMJ in aged mice (453). More detailed morphological and functional studies of the NMJs in the PGC1α transgenic mice at young age show that NMJs are altered to a phenotype characteristic of slow muscle fibers and that those changes also affect the presynaptic terminals (12). It is also interesting to note that PGC1α has been implicated to act as a cotranscriptional regulator of GABPα/GABPβ in the expression of synaptic genes at the NMJ (173). In summary, overexpression of PGC1α phenocopies, at least in part, endurance training and prevents some of the detrimental effects of aging including fragmentation of the NMJ. Conversely, a decrease in mitochondrial function in the course of aging may also cause changes in NMJ structure observed at higher age.

Another signaling pathway that is perturbed during aging and thus may participate in sarcopenia is the mTOR pathway. As described in section VI, mTORC1 is a central hub that integrates growth factor signaling and the nutrient status (in particular amino acid availability) to control cell growth. Intriguingly, inhibition of mTORC1 has been implicated in longevity. For example, mutations that render the TOR pathway hypoactive increase lifespan in yeast and make the mutants not responsive to the life-prolonging effect of CR (217). A similar positive effect on lifespan is seen with mutants for Sch9, the yeast ortholog of S6K. TOR mutants also live longer in Drosophila (220) and C. elegans (440). Most importantly, rapamycin fed to mice has a life- prolonging effect (176), which is even seen when treatment starts only at the age of 9 mo (303). Finally, body-wide deletion of the mTORC1 target S6K1 also extends lifespan in mice (393). Thus the evidence is strong that mTORC1 affects longevity, but it remains to be determined which age-related diseases are mitigated by mTORC1 inhibition.

### E. mTORC1 and Sarcopenia

Although the onset of sarcopenia was not investigated in detail in the rapamycin-treated mice, age-related loss of locomotory activity is delayed in male mice treated with rapamycin (303), indicating a beneficial effect on muscle. It appears paradoxical that rapamycin would prevent sarcopenia as mTORC1 signaling is known to promote cell growth and to be required for compensatory hypertrophy in response to hindlimb suspension (48). Moreover, skeletal muscle fibers deficient for raptor (i.e., inactive mTORC1) do not become hypertrophic in functional overloading experiments (37). Activation of the mTORC1 pathway by infection of skeletal muscle with IGF1-expressing viruses (27) or transgenic expression of IGF1 (316) induces a strong muscle hypertrophy in young and old mice. Finally, expression of a constitutively active form of Akt/PKB also induces muscle hypertrophy (48, 204, 244). In humans, rapamycin given before resistance exercise, which is a well-established stimulus to induce muscle hypertrophy, prevents the increase in muscle protein synthesis (120). These data have led to the hypothesis that IGF1-P3K-Akt/PKB-mTOR signaling might be lowered at high age. Indeed, mTORC1 signaling and subsequent increase in muscle protein synthesis in response to resistance exercise is blunted in more than 70-yr-old people compared with young adults (145). These observations have led to the suggestion that local application of IGF1 and thus activation of mTORC1 might be a valid strategy to counteract sarcopenia (70).

In contradiction to this suggestion, there is no significant change in the activation state of IGF1 or Akt/PKB at old age. In fact, phosphorylation of S6, a downstream target of mTORC1, is increased in the muscles of old humans and of old mice compared with young adults (378). These data indicate that mTORC1 might rather be hyper- than hypo-active at high age. Consistent with the notion that hyperactivation of mTORC1 might boost and not prevent sarcopenia, long-term activation of this pathway in skeletal muscle in mice is detrimental (83, 378). In fact, mice where mTORC1 is constitutively active develop a myopathy at the age of ~1 yr (83). The observed phenotype is reminiscent of
sarcopenia in several aspects, including the lowering of muscle mass and size (37), and the lower specific muscle force (83). Several lines of evidence indicate that the phenotype is, at least in part, caused by the block of autophagy induction in skeletal muscle (84), a process that is well known to be involved in longevity (307). Consistent with this interpretation, blockade of autophagy by muscle-specific elimination of Atg7 results in a late-onset myopathy (285). Interestingly, autophagy is strongly induced by endurance exercise (178) and CR (459), which are both paradigms that ameliorate sarcopenia. Thus the “anti-aging” effect of CR and endurance exercise might be based on its dampening mTORC1 signaling and thereby increasing autophagy flux to allow clearance of nonfunctional proteins and organelles (e.g., mitochondria). The recent finding that rapamycin lowers rather than increases the atrophy of muscle upon denervation (417) supports the notion that sustained activation of mTORC1 might accelerate the development of sarcopenia.

Although little is known whether changes in mTOR signaling also affect the NMJ directly, recent work indicates that the turnover of the AChRs is affected by the overall metabolic state of the muscle. For example, fasting and denervation also affect the overall turnover of AChRs by increasing the number of AChR-containing endosomes/lysosomes (223). Thus changes in muscle metabolism that influence mTOR signaling and muscle homeostasis may well also affect NMJ integrity.

VIII. CONCLUSIONS AND PERSPECTIVE

The essential role of neuromuscular transmission for skeletal muscle contraction (e.g., respiration) warrants a highly reliable and redundant system to build and maintain the NMJ. In the past two decades, we have witnessed the rapid progress in the understanding of the molecular basis of NMJ development. These findings are largely corroborated by genetic evidence showing that many of the key molecules involved in NMJ development are also the cause of CMS or targets of sporadic MG. Because of the vital function of neuromuscular transmission, these diseases are, however, very rare.

The findings that the neuromuscular system undergoes major changes during aging (see also **FIGURE 7A**) have led to a new wave of molecular studies investigating the role of the genes involved in NMJ development in the course of aging. In our view, it will be of utmost importance to first firmly establish that changes in neuromuscular transmission indeed contribute to sarcopenia as sarcopenia can arise from many different causes. For example, progressive denervation of muscle can be the result of sporadic death of indi-
vidual motor neurons. Longitudinal studies indicate that there is an up to 50% loss of motor neurons at high age (427). This process leaves muscle fibers transiently denervated (FIGURE 7) and evokes sprouting by the surviving motor neurons, which increases the size of the motor units. As sarcopenic patients indeed show an increase in motor unit size (119), motor neuron loss may also contribute to the loss of muscle mass although reinnervation of muscle by the surviving motor neurons may delay this process. In addition to this presynaptic effect, there is plenty of evidence that changes in the function and the metabolism of skeletal muscle contribute to sarcopenia (FIGURE 7). Thus current evidence strongly indicates that sarcopenia is caused by pathological processes in both pre- and postsynaptic cells.

Another exciting new avenue is the observation that pathways and behavioral paradigms involved in longevity, such as CR and endurance exercise, also show antisarcopenic effects in animal studies and affect NMJ structure. Moreover, there is also evidence that the molecular pathways mediating the positive effects of CR or endurance training also affect sarcopenia and NMJ structure. These observations raise the exciting possibility that identifying the molecular signatures in skeletal muscle that affect whole-body metabolism may allow identifying pathways that are also beneficial to the neuromuscular system. Finally, recent heterochronic parabiosis experiments, where the circulation of two animals of different age is joined, provide evidence that factors circulating in the blood may counteract aspects of aging in muscle (100).

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Address for reprint requests and other correspondence: M. A. Rüegg, Biozentrum, Univ. of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland (e-mail: markus-a.ruegg@unibas.ch).

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